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# Biological and environmental factors associated with the stress response in infancy. The role of attachment and genetics.

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I, Alessandra Frigerio, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## ABSTRACT

The importance of understanding which environmental and biological factors are involved in determining individual differences in physiological response to stress is widely recognized, as the impact of stress on physical and mental health cannot be ignored. Many researchers believe that our ability to cope with stress originates in infancy through the interaction between our experiences and genes. Several studies have shown how attachment relationships as well as some temperamental traits play a significant role in modulating Hypothalamic Pituitary Adrenal (HPA) axis reactivity to the stress of separation from the mother, as indexed by salivary cortisol. However, no published studies have investigated their effects on Sympathetic Adreno Medulla (SAM) system reactivity, as indexed by salivary alpha amylase which was recently found to be a marker of SAM activity. Moreover, the contribution of genetics in predicting salivary cortisol and alpha amylase response to stress in infants has not been investigated yet. In the present study, the child-mother attachment relationship, some genetic polymorphisms (DRD4, DRD4/521, COMT and 5-HTT), and temperamental traits were tested as predictors of both physiological markers during the Strange Situation (SS) procedure in an Italian sample of around 70 healthy infants aged 12 to 18 months. HPA and SAM activity was predicted by a larger number of gene x environment interactions in comparison with the number of separate constitutional (genes and temperament) and environmental (attachment patterns) factors. These results help to disentangle the role played by both biological and environmental factors in determining individual differences in stress response in infancy. The results also shed light on the suggestion that HPA and SAM systems are likely to have different characteristic responses to stress and associations with behaviour.

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# CHAPTER 1

# BIOLOGICAL AND ENVIRONMENTAL FACTORS ASSOCIATED WITH THE STRESS RESPONSE IN INFANCY

This literature review will examine the role that environmental and genetic factors play in infants' stress responsivity. Attachment will be focused on as a key candidate environmental individual difference variable (Bokhorst et al., 2003) that may confer resiliency against stress in securely attached children. A range of recently studied single gene polymorphisms, as well as measures of temperament, will be considered as indices of potential genetic influence on stress responsitivity.
The review begins by introducing the organization of the neurobiological systems mediating stress responses, and studies of the hormonal stress response in adults and children are outlined in detail (part 1). Following this, attachment theory is reviewed and its main features, developmental course and role as a coping process for infants are described (part 2 a); then, other environmental factors related to inadequate parental care, such as maltreatment, assumed to play a significant role in determining individual differences in physiological stress response are illustrated (part 2 b). The last section of this review (part 3) presents the contribution of single genetic polymorphisms (part 3 a) as well as maladaptive temperamental traits (part 3 b) as significant predictors of the activity of the stress-related systems.

## PART I. BIOLOGICAL SYSTEMS AND MARKERS OF THE STRESS RESPONSE

### THE PSYCHOBIOLOGY OF STRESS RESPONSE

The stress response has at least two principal components: one involves corticotropin-release hormone, activation of the hypothalamic pituitary adrenal axis (HPA), and the secretion of glucorticoids (e.g. cortisol) into circulation, whereas the second involves activation of the locus coeruleus/autonomic (sympathetic) nervous system (ANS) and the release of catecholamines (e.g. norepinephrine) into the blood stream (Chrousos and Gold, 1992). These two systems will be examined in the following paragraphs after a brief synthesis on concepts of stress and stressors as well as a general description of mechanisms and phases of the stress response.

**Stress and stressors.** Hans Selye (1956) defined stress as the non-specific response to any demand, whether it is caused by, or results in, pleasant or unpleasant conditions. Within the general concept of stress, a distinction between distress (from the Latin dis = bad), and eustress (from the Greek eu = good) is made but, according to this definition, stress as such is seen as all-inclusive, embodying both the positive and the negative aspects of these concepts. According to Selye, during both eustress and distress the body undergoes virtually the same nonspecific responses to the various positive or negative stimuli acting upon it.

Later definitions have extended Selye's view of stress, as the non specific response to any demand, providing more emphasis on the role played by individual characteristics in stress response. Thus, stress is defined as any challenge to homeostasis of an individual that requires an adaptive response (Newport and Nemeroff, 2002). Another clear example of this new perspective is represented by Ivancevich and Matteson's (1980) definition of stress as "an adaptative response, mediated by individual characteristics and/or psychological processes, which is a consequence of any external action, situation or event that places special physical and/or psychological demands upon a person (p.9)".

A stressor can be defined as a change in the environment that is sensed by an organism, is aversive and potentially harmful to that organism and elicits acute and/or chronic responses (Ottenweller, 2000). Stressors can be aversive exteroceptive (e.g., eletric shock, cold, but also several psychosocial aversive situations in humans, such as perfomance tasks, public speech) and interoceptive (e.g. pain) stimuli, varying by intensity and duration. However, which characterists of stressors are able to trigger a stress response is still debated. According to Selye's perspective all stressors would elicit the same physiological reaction, whereas other authors argue that just extreme or prolonged stressful conditions can provoke a reaction of the organism. Recently, Dickerson and Kemeny (2004) have reported how the socioevaluative threat and uncontrollability of the stressor may be stronger elicitors than others of physiological responses.

A stress response in turn consists of a complex pattern of physiological, behavioural, cognitive, and/or emotional components.

**General Adaptation Syndrome.** General Adaptation Syndrome (GAS), descripted by Selye (1952) with the aim to interpret the effects of stress, is the pool of multifactorial responses following a stressor aimed to adjust the organism to changing events in order to overcome it or to tolerate it. In other words, the general adaptation syndrome can be interpreted as the pool of biological reactions of an organism subjected to stressful events organised around maintaining internal conditions as constant as possibile to those preceeding the applied stimulus. The GAS, therefore, is an homeostatic mechanism wherein 3 following phases can be identified:

1. alarm reaction phase
2. adjustment or resistance phase
3. exhaustion phase

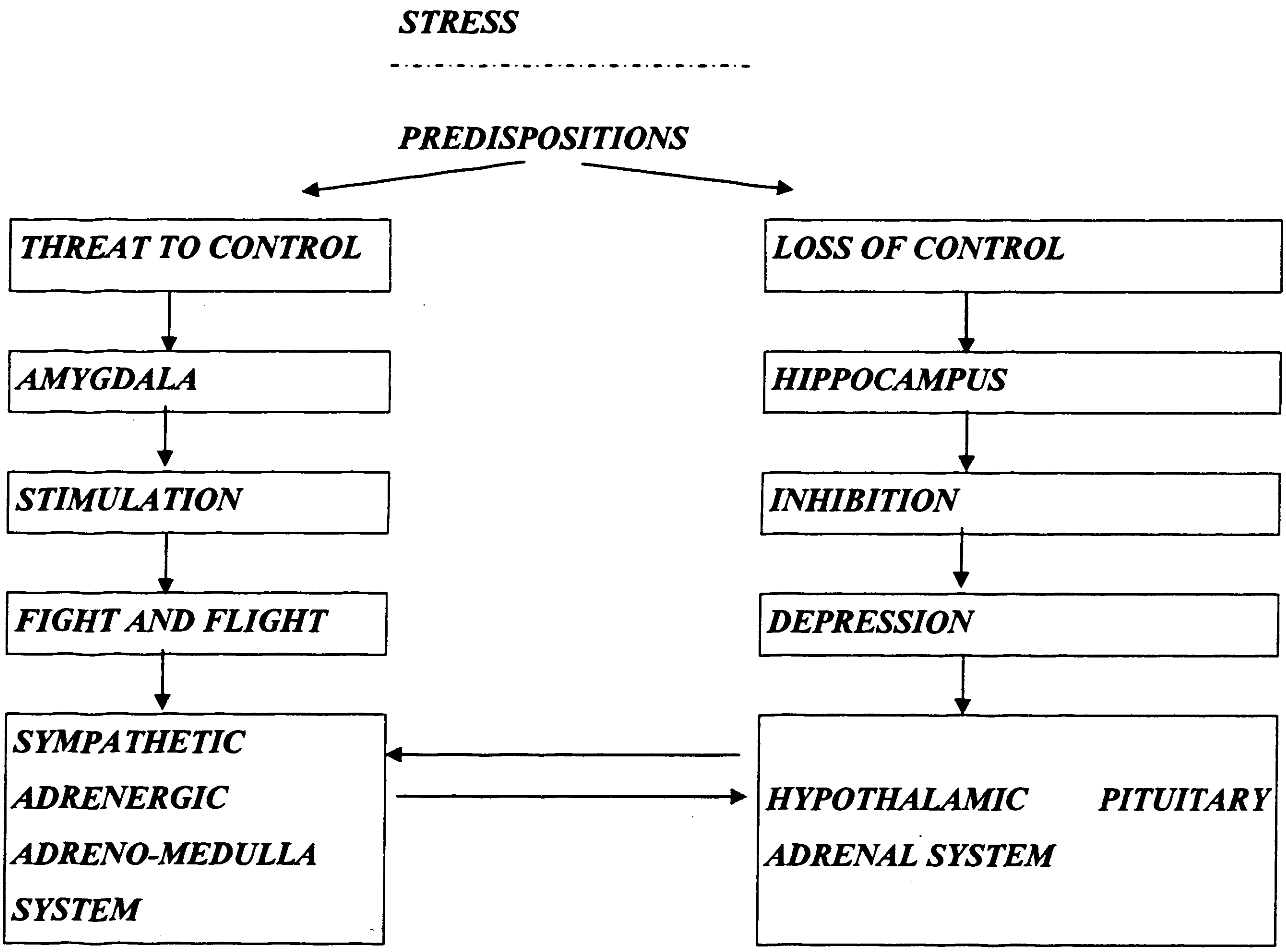
According to Selye's model, the *alarm phase* is composed of all those non-specific reactions subsequent to a stress which have a rapid and violent action toward which the organism is not qualitatively and quantitatively adapted. The alarm reaction can be divided into two phases which follow one another: the shock phase, wherein the organism is passively subjected to the stimulus and the contro-shock phase corresponding to the mobilization of the organism's non-specific responses.

The *adjustment phase* happens when the organism has been adapting to the stressor; it is also sometimes defined as the *resistance phase* because the organism's resistance toward the stimulus increases in this period. The pool of non-specific systemic responses, determined by prolonged stressors, are activated in this phase, allowing the organism to adapt.

The *exhaustion phase* corresponds to the period in which the ability to manage the adjustment exhausts or fails and the organism gives way to the aversive conditions. It can appear more or less late according to the organism's coping resourses and the intensity of the stressful event. It may also be absent when the stress terminates within a given timeframe. This pool of reactions is thought to have evolved during philogenesis in a species-specific manner. The same stressful event can produce - on the basis of genetic, imprinting, educational, experiental and other predispositions - two distinct types of phenomena: the fight-flight response or 'depression', meaning a relatively slow-acting biological process of adaptation (Jannini et al., 1988). The first response is regulated by the neuroendocrine centre of the amygdala and is activated by the sympathoadrenergic-adrenomedulla system. The second response, instead, is controlled centrally by the hippocampus septum and is characterized by the activation of the hypothalamic pituitary adrenal axis (see figure 1.1).

Figure 1.1

*Organism's reactions in response to a stressful event (from Jannini et al., 1988).*




In humans, the choice of which of the two pathways does not seem to be dependent on the nature of the stimulus but how the stimulus is read and interpreted (e.g. Jannini et al., 1988).

**Autonomic and metabolic phase.** The autonomic phase is the first mechanism activated by the stressful event and it is characterized by the activation of the adrenal medulla and by the neuropituitary. In this phase catecholamines and vasopressin are released in direct proportion to the intensity of the stimulus. The central mechanism of this phenomenon is to be found in the activation of the sympathetic nervous system. The adrenal medulla is activated and the circulating catecholamines are produced by this rapid response. The autonomic phase corresponds to the alarm phase of the general adaptation syndrome, with its rapid and effective response characteristics. The metabolic phase follows the autonomic one. The metabolic phase is slower than the previous one as it can be chronologically evaluated by minutes, and it is characterized by the anteropituitary hormonal response.

## HYPOTHALAMIC PITUITARY ADRENAL (HPA) AXIS AND THE ROLE OF CORTISOL

### HPA Axis Functioning

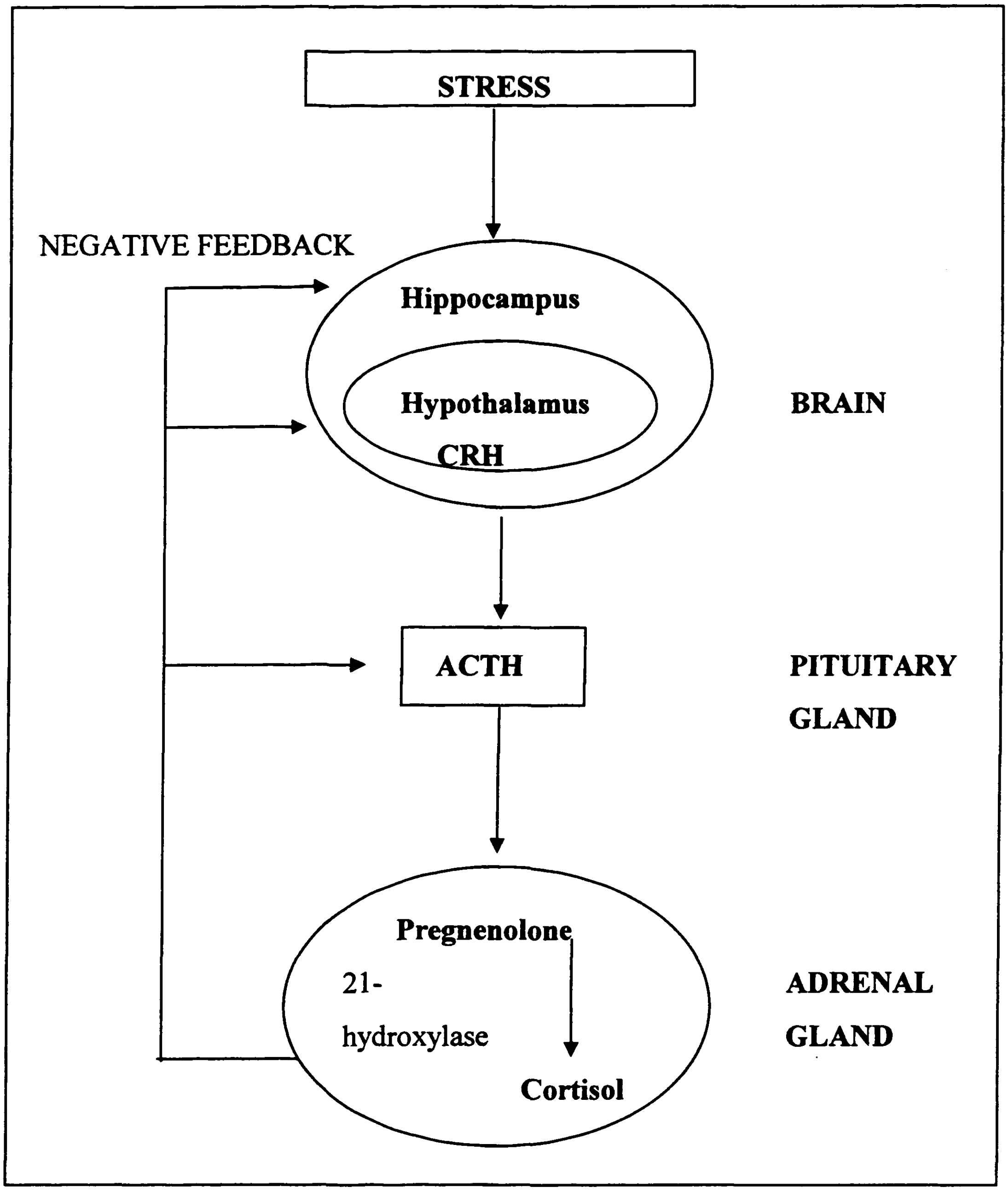
The hypothalamic pituitary adrenal axis (HPA) is one of the stress response systems of the body, which consists of the hypothalamus, the pituitary gland and the adrenal gland. The HPA axis activates and coordinates the stress response by receiving and interpreting information from other areas of the brain (the amygdala and the hippocampus) as well as from the autonomic nervous system (Holsboer, 2001). It can be activated by a multitude of stressors, from physical insults such as surgery to psychological stimuli such as social stress, subordination, or perceived threat (Lopez et al., 1999).

A hormonal cascade is initiated in response to the stressor with the release of corticotropin releasing hormone (CRH) from the hypothalamus, which stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the pituitary gland. ACTH then triggers the breakdown of pregnenolone within the adrenal cortex into the glucorticoid cortisol, which is subsequently secreted into the blood circulation. Glucorticoids provide negative feedback at the level of the hypothalamus, the pituitary, and the hippocampus, thereby shutting off the stress response (see Fig. 1.2).

The HPA axis provides the adaptive mechanisms needed to maintain homeostasis in times of increased stress. These responses are protective in acute situations but they can be damaging if the hormones are overproduced or dysregulated over long periods of time (McEwen, 2002).

Figure 1.2

*The HPA axis (from Shea et al., 2004)*



## Salivary cortisol

The Hypothalamic-Pituitary-Adrenocortical system produces steroid hormones termed glucorticoids (GCs) of which cortisol is the predominant one in humans. In the early 1980s, the introduction of new assay techniques allowed the measurement of salivary cortisol giving researchers the possibility of investigating this important indicator of stress easily and non invasively.

Cortisol is a corticosteroid hormone that is synthesized from progesterone, which is made from the precursor of all steroid hormones called pregnenolone. The conversion involves hydroxylation of C-11, C-17 and C-21. The synthesis takes place in the *zona fasciculata* of the cortex of the adrenal glands. While the adrenal cortex also produces aldosterone (in the *zona glomerulosa*) and some sex hormones (in the *zona reticulosa*), cortisol is its main secretion. In normal conditions, cortisol has widespread actions which help restore homeostasis after stress. It acts as a physiological antagonist to insulin by promoting gluconeogenesis, breakdown of lipids, and proteins, and mobilization of extrahepatic amino acids and ketone bodies. This leads to increased blood glucose concentrations, resulting in increased glycogen formation in the liver. It also increases blood pressure. In addition, immune and inflammatory cells have their responses to stress attenuated by cortisol, and the hormone thus lowers the activity of the immune system. Bone formation is also lowered by cortisol.

As previously described, cortisol is produced and released by the cortex of the adrenal glands in response to the release into general circulation of adrenocorticotropic hormone (ACTH). GCs in circulation enter into the cytoplasm of cells throughout the body and brain where the hormone interacts with its receptors. The activated receptors then enter the nucleus of the cell where they regulate the transcription of genes with GC responsive elements (GREs) in their promoter regions (Sapolsky et al., 2000). The effects of GCs depend on the type of GC receptor that has been activated by the hormone. There are two types of receptors: mineralcorticoid receptors (MR) and glucorticoid receptors (GR). Both are responsive to GCs in the brain, while outside the brain GC effects operate almost exclusively through GRs. MRs and GRs mediate different, often opposing, functions: those mediated by MRs tend to promote processes that support physical and mental health, while those mediated by GRs tend to be suppressive in order to counter-regulate acute responses to stressors. For example, GR- mediated processes tend to reduce the processes involved in the formation of explicit memories which are activated as immediate responses to stressful events. The effects of increasing cortisol within basal levels are mediated by MR activation whereas the effects of increasing cortisol above basal levels are mediated by GR activation. However, elevations in cortisol to ranges associated with GR occupancy may be associated with healthy functioning if the stressor is acute and cortisol levels quickly return to baseline, while prolonged elevations may be associated with negative outcomes if they produce prolonged GR occupation.

Levels of GCs vary throughout the day under basal or non-stressed conditions: their peak is around the time of awakening in the morning while their lowest point is soon after the onset of night time sleep (Daly and Evans, 1974). This diurnal rhythm can be detected around the 6$^{th}$ post-natal week, becoming more reliable from day to day over the first months of life (Larson et al., 1998). Consequently, GR and MR are occupied at the peak of the daily cycle when GC levels are higher and are unoccupied at the end.

GCs are self-regulating in the sense that stimulation of GRs results in inhibitory signals to the paraventricular nucleus (PVN) of the hypothalamus that turn off production of CRH. The efficiency of this negative feedback varies with the time at which a stressor occurs during the diurnal rhythm: it takes smaller increases in GCs to stimulate GR-mediated negative feedback control at the peak of the rhythm, whereas GC increases tend to be larger near the end of the rhythm (Dickerson and Kemeny, 2004). Therefore, while studies conducted late in the day may be more capable of detecting reactivity of the system to stressors, studies of basal levels conducted earlier in the day may be more likely to detect stable and heritable trait components of the system.

Last, it is suggested that peak levels of salivary cortisol are reliably observed between 20 and 25 minutes following challenges; however, individual differences in the latency to display peak cortisol responses outside this range time have been reported among children as young as infants (Lewis and Ramsay, 2003; Goldberg et al., 2003).

# THE SYMPATHETIC NERVOUS SYSTEM AND THE ROLE OF SALIVARY ALPHA AMYLASE

The Sympathetic Nervous System (SNS) is part of the autonomic nervous system (ANS), which also includes the parasympathetic nervous system (PNS).
As previously described, the SNS activates the fight or flight response, also termed sympathetic-adrenal response. Briefly, the pre-ganglionic sympathetic fibres that end in the adrenal medulla (but also all other sympathetic fibres) secrete acetylcholine, which activates the secretion of adrenaline (epinephrine) and to a lesser extent norepinephrine from it. Therefore, this response, which acts primarily on the cardiovascular system, is mediated directly via impulses transmitted through the sympathetic nervous system and indirectly via catecholamines secreted from the adrenal medulla.

**Organization.** Sympathetic nerves originate inside the vertebral column, toward the middle of the spinal cord in the intermediolateral cell column (or lateral horn), beginning at the first thoracic segment of the spinal cord and extending into the second or third lumbar segments. Because its cells begin in the thoracic and lumbar regions of the spinal cord, the SNS is said to have a *thoracolumbar outflow*. Axons of these nerves leave the spinal cord in the ventral branches (rami) of the spinal nerves, and then separate out as 'white rami' (so called from the shiny white sheaths of myelin around each axon) which connect to two chain ganglia extending alongside the vertebral column on the left and right. These elongated ganglia are also known as 'paravertebral ganglia' or 'sympathetic trunks'. In these hubs, connections (synapses) are made which then distribute the nerves to major organs, glands, and other parts of the body.
In order to reach the target organs and glands, the axons must travel long distances in the body, and, to accomplish this, many axons link up with the axon of a second cell via synaptic connections.

In the SNS and other components of the peripheral nervous system, these synapses are made at sites called ganglia. The cell that sends its fibre is called a *preganglionic* cell, while the cell whose fibre leaves the ganglion is called a *postganglionic* cell. As mentioned previously, the preganglionic cells of the SNS are located between the first thoracic segment and the second or third lumbar segments of the spinal cord. Postganglionic cells have their cell bodies in the ganglia and send their axons to target organs or glands.

The ganglia include not just the sympathetic trunks but also the superior cervical ganglion (which sends sympathetic nerve fibres to the head), and the celiac and mesenteric ganglia (which send sympathetic fibres to the gut).

**Information transmission.** Messages travel through the SNS in a bidirectional flow. Efferent messages can trigger changes in different parts of the body simultaneously. For example, the sympathetic nervous system can accelerate heart rate; widen bronchial passages; decrease motility (movement) of the large intestine; constrict blood vessels; cause pupil dilation, piloerection (goose bumps) and perspiration (sweating); and raise blood pressure. Afferent messages carry sensations such as heat, cold, or pain. The first synapse (in the sympathetic chain) is mediated by nicotinic receptors physiologically activated by acetylcholine, and the target synapse is mediated by adrenergic receptors physiologically activated by either norepinephrine or epinephrine. An exception is with sweat glands which receive sympathetic innervation but have muscarinic acetylcholine receptors which are normally characteristic of the PNS. Another exception is with certain deep muscle blood vessels, which have acetylcholine receptors and which dilate (rather than constrict) with an increase in sympathetic tone. Table 1.1 describes the main functions of sympathetic and parasympathetic nervous systems.

Table 1.1

*Main functions of sympathetic and parasympathetic nervous systems*

| ***Functions of Sympathetic System*** | ***Functions of Parasympathetic System*** |
|---|---|
| Dilates pupil | |
| Inhibits flow of saliva | Stimulates flow of saliva |
| Accelerates heart beat | Slows heart beat |
| Dilates bronchi | Constricts bronchi |
| Inhibits peristalsis and secretion | Stimulates peristalsis and secretion |
| Conversion of glycogen to glucose | Stimulates the release of bile |
| Secretion of adrenaline and norepinephrine | |
| Inhibits bladder contraction | Contracts bladder |

**Key role for cathecolamines in stress.** Catecholamines (dopamine (DA), adrenaline and norepinephrine (NE)) are among the first molecules to show a response to stressors and are crucial in stimulating action in response to a perceived threat. In the periphery, the sympathoadrenal system is responsible for the rapid increase in plasma concentrations of NE, adrenaline and co-localized neuropeptides triggered by stress. Adrenaline, the main hormone released by the adrenal medulla, and NE, released mainly from sympathetic nerve endings and partly from the adrenal medulla, activate the heart and skeletal muscles to prepare for the "fight or flight" response during stress. Similarly, stress activates catecholaminergic areas in the brain, which have a widespread influence on neuronal responses to stressors. For example, in response to an acute stressor, catecholamines stimulate some limbic regions, such as the hippocampus, amygdala and hypothalamus, whereas other regions, such as the higher cognitive centres in the prefrontal cortex, are inactivated. Catecholamines are crucial in mediating stress-induced increases in vigilance and alertness.

## Salivary alpha amylase

Salivary alpha amylase, an enzyme produced by the salivary glands, has increasingly being seen as a non-invasive marker of SNS (Granger et al., in press). The activation of SNS affects the release of catecholamines such as epinephrine (EPI) and norepinephrine (NE) from nerve endings in tissues and glands. Beta-adrenoreceptors are present in the salivary gland, glandular duct cells and vascular bed of the salivary glands (Nederfors and Dahlof, 1992) and influence salivary α-amylase levels in response to SNS activation.

Salivary α-amylase is an enzyme rather than a hormone, immunoglobulin, or metabolite, officially classified as family 13 of the glycosyl hydrolases. The structure is an 8 stranded α-β barrel containing the active site, interrupted by a ~70 a.a. calcium-binding domain protruding between beta strand 3 and α helix 3, and a carboxyl-terminal Greek key β-barrel domain. It is produced in the oral mucosa by the salivary gland and, under conditions of normal oral health, it is present in saliva in relatively high concentrations. The digestion of macromolecules such as carbohydrates and starch jointly with the prevention of bacterial attachment and bacterial clearance from the mouth are respectively the primary and secondary biological function of salivary α-amylase.

At birth, salivary α-amylase is not present, as newborns are primarily protected against foreign antigens by passive immunity received from their mothers' colostrum or breast milk. The age of onset in the rise of salivary α-amylase (9 – 21 months) corresponds to the introduction of solid foods in the diet and the emergence of dentition and it reaches maximum levels by 5-6 years of age (O'Donnell and Miller, 1980). Recently, some authors reported individual differences in alpha amylase activity associated with age (El-Sheikh et al, 2005; Stroud et al., 2005, Susman et al., 2006). Moreover, a recent study found that salivary α–amylase activity shows a distinct diurnal profile with a trough in the morning and a steady increase of activity during the day in a large sample of adults (Nater et al., 2007). However, no data is yet available about the presence of a similar alpha amylase diurnal pattern in childhood.

The idea of considering salivary α-amylase as a good physiological parameter of the stress response began in the late 1990s when a series of studies (Chatterton et al., 1996, 1997; Skonsnik et al., 2000) which evidenced a strong positive association between salivary α-amylase levels and the SNS component. Specifically, salivary α-amylase concentrations were associated with baseline plasma catecholamine levels, particularly NE as well as NE change in response to stress (Chatterton et al., 1996; Rohleder et al., 2004); furthermore, the administration of the adrenergic blocker propranolol was very recently proved to inhibit stress-related increases in α-amylase levels (van Stegeren et al, 2006). However, although it seems reasonable to consider salivary α-amylase as a correlate of the adrenergic component of the stress response, it would be not cautious to interpret it as a specific and direct marker of NE (Granger et al., 2006).

## THE RELATION BETWEEN THE HPA AND SNS SYSTEMS

The HPA axis and the SNS are biologically intertwined (Chrousos and Gold, 1992; Young et al., 2005). As Chrousos and Gold (1992) discuss extensively, the locus coeruleus – norepinephrine (LC-NE) and corticotrophin releasing hormone (CRH)/HPA components of the stress response system connect at several brain sites. For example, neurons that secrete CRH project from the lateral PVN in the hypothalamus to sympathetic hindbrain regions (Chrousos and Gold, 1992). Administration of CRH increases catecholaminergic activity and NE levels (Brown et al., 1982; Dunn and Berridge, 1990). CRH antagonists cause reduced responsiveness in the LC-NE system (Dunn and Berridge, 1990). Catecholaminergic pathways project from the LC to the PVN (Brown et al., 1982; Chrousos and Gold, 1992; Dunn and Berridge, 1990). NE stimulates the release of CRH in the PVN (Brown et al., 1982; Dunn and Berridge, 1990), and beta-adrenergic blockers reduce the behavioural consequences of CRH (Chrousos and Gold, 1992; Dunn and Berridge, 1990). Although the two systems thus are interconnected physiologically, they appear to have different characteristic responses to stress and associations with behaviour. For example, they habituate at different rates (Schommer et al., 2003). In addition, some research suggests that the HPA axis is activated by negative affect associated with stress, including fear and frustration, whereas the SNS is related to effort and is valence non-specific (Frankenhaeuser, 1982; Lovallo and Thomas, 2000). Furthermore, salivary α-amylase and cortisol should be considered as two separate and independent stress response indexes as their levels at baseline, after a challenge or during a recovery do not seem to be correlated (Chatterton et al., 1996; Gordis et al., 2006; Granger et al., In press; Nater et al., 2006).

Therefore, while it is clear that the HPA axis and the SNS work in coordination to generate the physiologic changes associated with the stress response, the exact nature of the coordination (e.g. additive or interactive; opposing or complementary) is a subject of debate. Henry (1992) speculates that SNS activity increases in response to challenges that are perceived as manageable or controllable, whereas an HPA response is more likely during emotionally distressing or uncontrollable situations.

## MODELS OF STRESS RESPONSE MECHANISMS

Various models of stress have been elaborated for explaining the role played by different stressors and different individual characteristics in triggering and modulating the physiological response to stress.

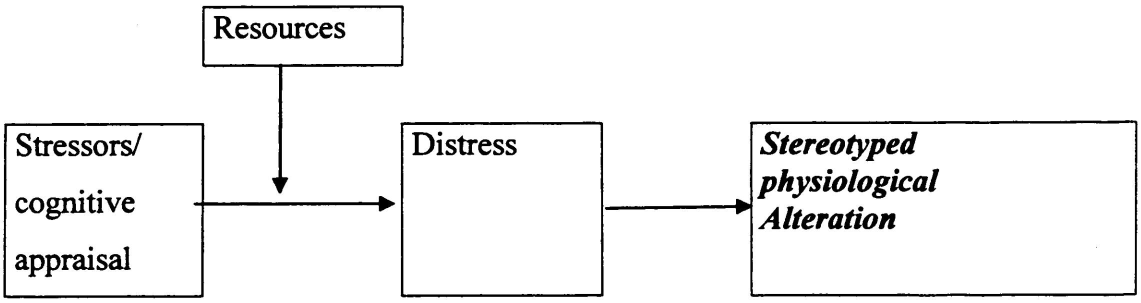
As previously described, Hans Selye argued that the physiological response to stressful circumstances is non specific in the sense that all stressors, whether physical or psychological, are capable of triggering physiological changes. Newer versions of this *generality model* suggest that if stressful circumstances lead to the experience of distress (or perceived stress), then a stereotyped set of physiological changes will be elicited. The significant role of psychological and environmental factors that can moderate the relationship among stressor exposure, distress and physiological activation is also emphasized by these modern versions of the generality model. However, the relationship between stressors and physiological reactions still remains the same.

According to others, instead, the specificity of this relationship (*specificity model*) is becoming evident. An integrated specificity model of stressor physiology was advocated by Weiner (1992) who argued that "organisms meet...challenges and dangers by integrated behavioral, physiological patterns of response that are appropriate to the task" (p. 33). Kemeny (2003) has further specified stated that "*both behavior and physiology are parts of an integrated response to address a specific environmental condition* (see Fig. 1.3), *and specific conditions or environmental* *signals elicit a patterned array of hormonal and neural changes that are designed to ready the organism to deal with the specific nature of the threat*".

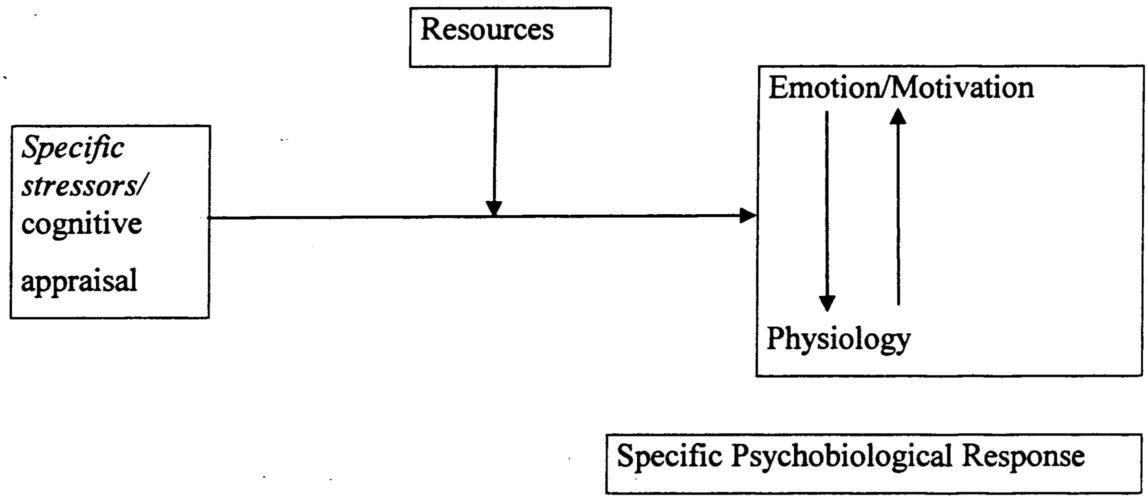
Figure 1.3

*The generality and integrated specificity models of stress (from Kemeny, 2003)*

***The generality model of stress***




***The integrated specificity model of stress***




A review of 208 studies (Dickerson and Kemeny, 2004) which investigated plasma or salivary cortisol responses to acute psychological laboratory stressors in healthy adults supports the specificity model of stress. This review shows that, like physical stressors (e.g., electric shock), psychological stressors can activate the HPA axis although not all of them can provoke this system. Specifically, Dickerson and Kemeny (2004) reported that significant elevations in cortisol levels were triggered by performance tasks characterized by socio-evaluative threat and/or uncontrollability, with the largest increases found for tasks which contained both elements. Furthermore, the overall magnitude of time to recovery as well as ACTH responses were also affected by these conditions. These findings strongly support the authors' model on the basis of which the cortisol system is activated in goal-relevant situations (motivated performance tasks) when a central goal is saliently threatened (social-evaluative threat) and the process for attaining this goal is impeded (uncontrollability). Consequently, these findings are clearly inconsistent with the notion that all psychological stressors elicit cortisol responses and they also call into question the presence of a nonspecific physiological response to all stressors that include HPA activation (e.g. Kemeny, 2003; Selye, 1956).

In the following paragraphs, the role played by the quality of care in promoting differences responses in HPA and SNS activation will be presented.

## STUDIES ON CORTISOL REACTIVITY IN CHILDHOOD: THE ROLE PLAYED BY SOCIAL REGULATION

Studies in rodents and primates show how social experiences during early development (Levine, 1994; Suomi, 1991) are likely to shape the responsivity and regulation of the Limbic-Hypothalamic-Pituitary-Adrenocortical (LHPA) system later in life. Caregiving behaviours like licking, grooming and arched back nursing seem to maintain a hyporesponsive period in rodents (Caldji et al., 1998; Suchecki et al., 1993) where the presence of the mother, in non-human primates, serves to buffer activity of the LHPA axis (Bayart et al., 1990). In humans, retrospective studies suggest that early adverse experiences predispose to dysregulated LHPA axis functioning in adulthood (Graham et al., 1999). A brief synthesis of the literature concerning the role played by maternal care in modulating adrenocortical reactivity in human infants will be examined in this paragraph. The focus will be on how early variations in care can explain individual differences in cortisol response in low and high risk samples.

There is empirical evidence (Gunnar et al., 1996; Larson et al., 1998; Ramsay and Lewis, 1994) that the high cortisol responsivity of newborns tends to diminish under conditions of sensitive and responsive caregiving by the end of the first year of life when many stressors trigger a reaction of the HPA axis only with difficulty. For example, physical exams were not found to increase cortisol levels among 3 months or older infants as they were found to do in younger infants despite the levels of behavioural distress being similar to those exhibited by the younger infants (Larson et al., 1998); significant elevations in cortisol were not more observed after two inoculations given for childhood immunizations at 12 and 18 months of age (Gunnar et al., 1996; Jacobson et al., 1994; Ramsay and Lewis, 1994). Furthermore, separation from parents in a strange setting with an unknown babysitter who had the instruction to respond to infant just when he/she was upset did not produce an increase of cortisol in 13 months infants (Gunnar and Nelson, 1994) and exposure to strange and novel events (e.g., live clown, toy robot) was not found to be associated with glucorticoid response in 12-to 18-months infants (Nachmias et al., 1996; Spangler and Schieche, 1998). Although the persistence in childhood development of this glucorticoid hyporesponsive period to mild stressful conditions is not well-known, some studies seem to support its prolongation at least in preschool children (deHaan et al., 1998; Gunnar et al., 1997; Kagan et al., 1987; Schmidt et al., 1997).

Gunnar and Donzella (2002) suggest that this glucorticoid hyporesponsiveness might be associated with the learned ability of infants, who experienced sensitive, responsive, and attentive caregiving, to anticipate that adults will protect them, thus allowing them to cope with threat. However, individual differences in cortisol response are found during this period when the quality of care the child receives is not of sufficient quality. The meaning of the term *quality of care* encompasses conditions such as the caregiver's availability, attention to the child, sensitivity to the child's needs, structuring of the environment, and responsiveness to the child's signals. In a study on the quality of babysitter care, Gunnar and colleagues (1992) found that distress at separation was related to increases in cortisol when infants were with the babysitter who was ignoring but not when they were with the responsive babysitter. Similarly, children who were in centre-based and family-based childcare of poorer quality (lower adult:child ratios and bigger group size) showed greater cortisol increases over the childcare day (Dettling et al., 2000). Furthermore, several studies reported that temperament interacts with the quality of care to predict glucorticoid response in the sense that adrenocortical activation was found to be present only in infants with high behavioural inhibition, fearfulness and poorer self-control and who were insecurely attached to their mothers. The same was not found in those who were temperamentally vulnerable but had a history of secure attachment (Gunnar et al., 1996; Nachmias et al., 1996; Schieche and Spangler, 2000; Schieche and Spangler, 2005).

Studies which have investigated cortisol activity in high risk samples where the quality of care was seriously compromised show not unequivocal results, with some of them reporting higher and others lower cortisol activation. In samples of children of depressed mothers, higher mid-morning home cortisol levels were found among three-year-old children compared to their peers with emotionally healthy mothers (Field, 1994; Hessl et al., 1998), maternal depression in the first year of the child's life was also associated with higher cortisol levels at age three (Hessl et al., 1998). Both elevated and suppressed activity of the HPA system has been found in samples of maltreated children, thus showing the complex and multi-faceted nature of this phenomenon (Cicchetti and Lynch, 1995). Finally, some studies looked into how the experience of institutional rearing can contribute to anomalies in HPA functioning. Gunnar and her colleagues (2001) found that orphanage-reared children had higher cortisol levels than not adopted children, and those who had lived in orphanages longer had higher cortisol response. There is also some evidence that for young children orphanage rearing fails to support a normal daytime rhythm in cortisol production (Carlson and Earls, 1997).

## STUDIES ON ALPHA AMYLASE REACTIVITY IN STRESSFUL PSYCHOLOGICAL CONDITIONS

The interest of researchers in salivary alpha amylase as a significant index of the sympathetic nervous system response to stress is recent. In spite of the relatively limited number of studies there is a growing empirical evidence that salivary α-amylase levels increases in response to both physical and psychological stress. Studies with adults and adolescents have shown how salivary α-amylase concentrations become higher in stressful physical conditions such as exercise, heat and cold stress and written examination (Chatterton et al., 1996; Chatterton et al., 1997; Skonisk et al., 2000), tasks as the Trier Social Stress Test (Nater et al., 2005, 2006) and situations of interpersonal (social rejection) stressors (Gordis et al., 2005; Stroud et al., 2005).

To date, few studies have investigated salivary α-amylase levels in infancy and childhood; moreover, none of them have been published yet. Granger and his colleagues (in press) reported the findings of these studies in a recent review, summarized below.

As far as the research carried out in samples of infants are concerned, there are just three studies which have looked into salivary alpha amylase responses to stress in this developmental period. Kivlighan and colleagues (2005) measured α-amylase levels in infants and their mothers before and after a series of tasks (i.e. arm restraint) designed to elicit emotional distress. Mothers were asked to watch without intervening. Whereas significant increases in salivary α-amylase levels following the stressor was found in mothers, no significant differences from pre- to post - task in alpha amylase concentrations emerged for the infants. However, 44.4% of infants showed an increase of α-amylase levels from pre- to- 20 minutes post task, when a 10% difference from pre-task was used as a criterion for change. Similarly, a recent study reported no significant differences, on average, in α-amylase concentrations in response to the strange situation procedure but significant differences related to infants' attachment patterns: children classified as avoidant showed higher mean levels of salivary alpha amylase than those classified as secure (Hill et al., 2006). Last, Shea and colleagues (Shea et al., 2006) reported positive correlations between maternal and infant salivary alpha amylase in response to some laboratory stressors (noise burst and infant arm restraint) in an investigation aimed to measure the effect of maternal depression/anxiety on the stress response.

There are also few studies which have measured salivary alpha amylase responses to stressful conditions in the preschool and school years. Mize and colleagues (Mize et al., 2005) found that 45% of preschool children experienced an increase of alpha amylase levels following a series of challenge tasks or games versus 37% of children who had an increase in their cortisol levels over the same assessments. Moreover, the authors reported that children who experienced a greater alpha amylase increase from pre-to post challenge had more illness and less close relationship with their teachers. The relationships between salivary cortisol, alpha amylase and behavioural problems, as well as cognitive and academic functioning were examined in two studies which used the same sample of 8-9 year-olds children (Buckhalt et al, under review; El-Sheikh et al, under review). Broadly speaking, lower salivary cortisol and alpha amylase levels in response to laboratory stressors were associated with the lowest levels of externalizing and internalizing behaviour problems, whereas the reactivity of these two physiological parameters was differently related to cognitive functioning for boys and girls. Last, Kivlighan and colleagues (2006) found that 78 percent of a sample of 9 to 11 years children showed a salivary α–amylase change of at least 10% in response to the Trier Social Stress Test.

To sum up, the first studies on this "new" physiological parameter of the stress response in infancy and childhood seem to support its role in indexing physiological processes that affect, and are influenced by, psychological, behavioural and social processes (Granger et al., In press). Moreover and interestingly, individual differences in alpha amylase levels are likely to be related to characteristics of the relationships the children have with their caregivers. This latter evidence contributes to support data from cortisol studies which underline how social relations and maternal care can buffer physiological responses to stress in early development.

The next section of this chapter provides a general picture on infant attachment theory and research, as child-mother attachment relationship may represent a significant protective factor against stress in infancy. Moreover, extreme parental behaviours which can contribute to explain individual differences in physiological response to stress will be reviewed.

# PART II. ENVIRONMENTAL FACTORS ASSOCIATED WITH THE STRESS RESPONSE

## II. A) ATTACHMENT THEORY AND CHILD-MOTHER ATTACHMENT RELATIONSHIP IN INFANCY

The empirical roots of Bowlby's attachment theory originate in early studies aimed at investigating the psychological effects of deprived care (Goldfarb, 1943; Skodak and Skeels, 1949; Spitz 1945,1946), naturalistic observations of animal behaviour (Lorenz, 1935) as well as laboratory studies, wherein rearing conditions were experimentally manipulated (Harlow and Harlow, 1962; Hersher et al., 1958). These data along with Bowlby's clinical experience in a school for maladjusted children lead him to diverge from many of his psychoanalytic colleagues, who sustained the essential role of internal fantasies and impulses, for supporting the predominant one of childhood experience in the emotional development.

Attachment theory was first formulated in John Bowlby's paper (1958), entitled "The Nature of a Child's Tie to His Mother", where the child's tie to his mother is postulated as "*a product of the activity of a number of behavioural systems that have proximity to mother as a predictable outcome*" (Bowlby, 1969; pp179), and culminated in the well-known three volume work "Attachment and Loss" (Bowlby, 1969; 1973; 1980). According to Bowlby, all primates are born with the tendency to develop an attachment relationship with their mother or another significant caregiver, which has the survival function of protecting them from predators and other threats to survival. More specifically, proximity-seeking behaviour directed towards a protective figure - activated by threatening circumstances - which increases the possibilities of surviving for the young.

Therefore, attachment behaviour can be defined as "*any form of behaviour that results in a person attaining or retaining proximity to some other differentiated and preferred individual, who is usually conceived as stronger and/or wiser. This behaviour is shown whenever the person is scared, without energies or ill, and it is attenuated after having received comfort and care*" (Bowlby, 1982), while the organization of attachment behaviours within the individual defines the attachment behavioural system. During infancy, the object of this attachment is usually the mother or the person who consistently takes care of the psychological and physiological needs of the child. According to Bowlby (1969,1973), the child is born with a repertoire of behaviours (such as crying, clinging to, vocalizing etcetera), that are genetically determined, and which are aimed to guarantee him physical proximity to the adult. Such proximity represents, in fact, an essential condition for the child's survival whenever he is in a dangerous, or potentially dangerous, situation.

The activation of the attachment system can vary in intensity and accordingly in the terminating conditions: physical contact is needed when attachment is intensively activated, whereas active sight of mother or even sound of her is sufficient in the case when attachment is less intensively activated. Activating conditions can be classified into three categories (Bowlby, 1969):

1) condition of child;
2) behaviour of mother;
3) other environmental conditions.

Fatigue, hunger, pain, cold and illness are conditions of the child which activate his need to seek proximity to the mother. As far as maternal behaviours are concerned, any of them which discourage or threaten proximity can elicit attachment behaviours in child. Last, environmental conditions such as novelty, alarming events, approach and rebuff by strangers are capable of activating the attachment system.

When attachment behaviour in the infant is activated, the role of the caregiver in responding to the child's safety plays a critical and significant role. For example, every time a child has to cope with an unfamiliar situation he will experience less anxiety if he knows he can rely on the proximity of an attachment figure who will be available to help him if needed (Cassibba and D'Odorico, 2000). Consequently, the child's confidence in the availability of the caregiver to act as a "secure base" in conditions of perceived threat will make him more secure to explore the environment in comparison with a child who does not have such confidence (Cassibba and D'Odorico, 2000).

Accordingly, attachment theory suggests that the child builds a stable representation (Internal Working Model) of the caregiver's availability, responses to attachment signals and the conditions which can assure or threaten his safety (Bowlby, 1973). Internal Working Models (IWM) characterize, therefore, the representations which the child constructs about himself and his primary attachment figures, which reflect the history of their attachment interactions (Bretherton and Munholland, 1999). The characteristics of these representations are believed to account for observed individual differences in attachment behaviour and the relatively stable organization of attachment relationships over time (Cassibba and D'Odorico, 2000).

## THE DEVELOPMENT OF CHILD-MOTHER ATTACHMENT RELATIONSHIP AND THE INTERNAL WORKING MODELS

Bowlby and Ainsworth have described four phases of development of child-mother attachment relationship (Ainsworth, 1967, 1972; Bowlby, 1969), named: 1) the initial pre-attachment phase; 2) the phase of attachment-in-the-making; 3) the phase of clear-cut attachment; and 4) the phase of goal-corrected partnership (Ainsworth et al. 1972). Although no well defined boundaries among these phases can be identified, this division is considered useful and convenient by the authors.

### *1) The Initial Pre-attachment Phase (from birth to 8-12 weeks)*

From the beginning the baby is responsive to stimuli which come from people, although he is not capable of discriminating one person from another yet. The infant is equipped with a repertoire of signaling behaviours (such as crying, smiling and vocalizations) which serve to promote proximity and contact, and a few behaviours through which the baby can actively seek or maintain closer contact (for example, rooting, sucking, grasping and postural adjustment when held). This phase terminates when the baby is capable of discriminating among people and, in particular, of discriminating his primary caregiver(s) from others.

### *2) The Phase of Attachment-in-the-Making (from 3 to 6 months)*

During this phase the infant can clearly discriminate unfamiliar from familiar figures but also he becomes able to discriminate between one familiar figure and another. Consequently, he/she shows behaviours aimed at promoting proximity toward specific figures and not others; these preferred figures may also differ among them in the way they act to terminate an attachment behaviour, such as crying. In this phase, the child shows an expansion of the repertoire of active attachment behaviours such as the emergence of coordinated reaching.

*3) The Phase of Clear-Cut Attachment (from 6 to 36 months)*

This phase is characterized by the increasing ability of the child in seeking and achieving proximity and contact with preferred figures in an active way. Different from the previous phases, he is now capable of reaching proximity on his own account instead of relying on signaling behaviour to bring caregivers into proximity. The most significant behaviour for the attachment system among child's newly acquired ones is represented by locomotion thanks to which the child is able to approach, follow and greet a preferred figure when he/she is distant, departing or returning respectively. Other active contact behaviours such as clambering up, embracing, burying the face in the body of the attachment figure and so on contribute to the attachment behaviour system. Furthermore, signaling behaviours continue to be emitted and language begins to develop.

During this phase the child is not only active in seeking proximity/contact but also in exploring his environment, manipulating the objects he discovers, and learning about their properties. Moreover, in this phase infant's behaviour first becomes organized on a goal-corrected basis and then gradually becomes hierarchically organized in terms of overall plans.

*4) The Phase of a Goal-Corrected Partnership (approximately 3 years)*

In this phase the child is capable of seeing things from his mother's point of view, coming to infer something of his mother's set goals and something of the plans she adopts to achieve them. He becomes increasingly more able to understand what feelings and motives, set-goals and plans might influence her behaviour. Thanks to the acquisition of this new competence, mother and child develop a much more complex relationship, which Bowlby terms a "partnership". This partnership is "goal corrected" in the sense that child's attachment behaviour and his mother's reciprocal behaviour are flexible and hierarchical organized.

As briefly described in the previous paragraph, the child constructs internal working models, conceived as "operable" models of self and the others, from the experience had with the main attachment figures. These IWM serve to regulate, interpret and predict both the self's and the attachment figure's attachment-related behaviour, thoughts, and feelings (Bretherton and Munholland, 1999). They are more similar to an "event schemata" (e.g., Mandler, 1983) than a static "picture" and they include affective, "appraising" and cognitive components (Bowlby, 1973; Bretherton, 1985). Furthermore, in order to be adaptive the IWMs have to be 1) based on relevant data; 2) applicable to novel situations, and 3) continuously checked for consistency.

The development of the IWM of self and the attachment figures is complementary in the sense that the representation of the other as a figure capable of being responsive, supportive and protective provides to the child the conditions for building a self's representation as valued and loved; on the contrary, the experience of a parent not emotionally available, rejecting and/or interfering with child's need of exploration leads to the construction of a working model of self as devalued and incompetent. Once organized, they tend to operate outside conscious awareness and to resist dramatic change (Bowlby, 1980).

The role played by stable IWM in individual's development is a crucial issue in attachment research, which has been investigated in several studies. However, as an extensive literature review on this topic is beyond the aim of the present chapter just two crucial evidentia are reported:

1) There is relatively good evidence about the continuity of IWMs from infancy to childhood, adolescence and adulthood. Main et al. (1985) study was the first one wherein a significant association between security of attachment as assessed by the Strange Situation and later performance on representational attachment-related tasks was found. Recently, an elegant study of Carlson, Sroufe and Egeland (2004) showed continuity in relationship representation and developmental links between relationship representation and behaviour from infancy to late adolescence. In fact, significant correlations emerged among diverse representational assessments (e.g., interview, drawing, projective narrative) and between representational and concurrent observational measures of relationship functioning.

2) There is also evidence that parents' mental representations of their attachment experiences – assessed by the Adult Attachment Interview (AAI) - influence the quality of their child's attachment to them (Van IJzendoorn, 1995). According to

attachment theory, an important vehicle for transmitting parents' attachment representations to their children is the sensitive responsiveness of the caregivers to child's needs. Recently, however, other possible explanations of intergenerational attachment transmission including genetic influences have been suggested (i.e. Fox, 1995; O'Connor and Croft, 2001).

Despite the substantial continuity of IWMs across the lifespan, changes can happen in response to concrete experiences in childhood whereas the occurrence of "formal operations" during and after adolescence may allow an internally-driven change in an individual's working models. Changes in environmental conditions (such as divorce) can represent examples of concrete experience which can lead to a change in child's IWM, whereas positive attachment relationship with a partner or a therapist can bring a reconstruction of an originally insecure attachment representation (Bowlby, 1988; Guidano and Liotti, 1983; Main et al., 1985).

## ATTACHMENT PATTERNS AND THE STRANGE SITUATION

Individual differences in attachment patterns are most easily observed when the child is in a situation in which he has to balance the need for protection and comfort with the desire to explore the environment (Ainsworth et al., 1978). Individual differences in the quality of attachment relationships can be broadly separated into two categories: "secure" and "insecure" attachment relationships (Ainsworth et al., 1978; Bowlby, 1973). The two terms describe attachment behaviour that can be considered to reflect the infant's apparent perception of the availability of the caregiver if a need for comfort or protection should arise, and the organization of the infant's responses to the caregiver in light of those perceptions of availability. Security in the relationship with an attachment figure is considered to suggest that an infant is able to rely on that caregiver as an available source of comfort and protection if the need arises. On the other hand, infants with an insecure attachment relationship, who did not have experience of consistent availability of and comfort from the caregiver in case of need, are not able to direct appropriate attachment behaviours at their caregivers or are not easily comforted by them. In other words, insecure attachment patterns are considered to represent a less effective use of the parent as a source of comfort in times of stress.

The identification of meaningful individual differences in attachment behaviour is attributable to work by Mary Ainsworth who devised a standardized observational procedure, named the Strange Situation (Ainsworth et al., 1978), which has become the central tool for attachment research in infancy. It is a structured observational procedure consisting of seven 3-minute episodes in which the infant is alone, with mother, with a female stranger, or with both mother and stranger, in an unfamiliar setting. These episodes are ordered to increase stress in standardized increments that are manageable for the baby. There are two separations from the mother, one in which the infant is with the stranger and one in which the infant is first alone and then with the stranger before the mother returns (see Table 1.2).

Table 1.2 *Summary of the Eight Episodes of the Strange Situation (from Ainsworth et al., 1978)*

| Episode | Persons present | Duration | Brief description of the action |
|---|---|---|---|
| 1 | Mother/child & Visit coordinator | 30 sec | Visitor coordinator introduces mother and child to play room |
| 2 | Mother and child | 3 min | Child is led into the room and allowed to approach toys. Mother sits in her chair. Child explores |
| 3 | Stranger mother & child | 3 min | Stranger enters. First minute: stranger silent. Second minute: Stranger converse with mother. Third minute: Stranger approaches child. After 3 minutes mothers leaves unobtrusively |
| 4 | Stranger & child | 3min. or less [a] | **First separation episode**. Strangers's behaviour is geared to that of child |
| 5 | Mother & Child | 3 min or more [b] | **First reunion episode**. Mother greets and/or comforts child, then tries to settle him/her again in play. Returns to her chair. Stranger leaves after child is playing. Mother leaves after 3 minutes or at end of episode (knock on door), saying "bye bye" |
| 6 | Child alone | 3min. or less [a] | **Second separation episode** |
| 7 | Stranger & child | 3min. or less [a] | Continuation of second separation. Strangers enter and gears her behaviour to that of child |
| 8 | Mother & child | 3 min | **Second reunion episode**. Mother enters, greets child, then picks him/her up. Meanwhile stranger leaves unobtrusively. Mother eventually returns to her chair. |

a) Episode is curtailed if the baby is unduly distressed

b) Episode is prolonged if more time is required for the baby to become re involved in play

On the basis of this procedure, four different attachment styles have been identified:

- *Secure attachment*: infants are able to seek proximity with the caregiver in an active way during the reunion episodes and they are able to openly communicate their feelings during the separations from the caregiver. Once contact with the mother has been re-established, they are quickly comforted and are able to return to exploration of the environment.
- *Anxious-resistant attachment*: infants vigorously protest during the separations but show a combination of proximity seeking and contact resistance during the reunions. They are not consolable upon reunion and are unable to explore the environment for nearly all the duration of the procedure.
- *Avoidant attachment*: infants seem not to show distress during the separations and ignore and avoid the caregiver during the reunions, instead focusing their attention on other aspects of the environment.
- *Disorganized attachment*: infants show contradictory and unpredictable behaviours when they are in the presence of the caregiver, jointly with subtle indicators of apprehension, disorientation and disorganization of behaviour.

As previously described, infants who have experienced a sensitive caregiver are thought to develop a representation of the caregiver as a figure capable satisfying their attachment needs. Such "felt security" is thought to allow them to use the caregiver as a "secure base" during the Strange Situation (secure attachment). Infants, who have experienced inconsistent or inappropriate responses to their needs, will be unsure about the caregiver's availability as a "secure base". Consequently, they will be less prone to explore the environment and they will show more anxiety during the Strange Situation (anxious-resistant attachment). Infants, who have experienced a consistently unavailable or rejecting parent will develop an apparent deactivation of attachment behaviours. This reaction is thought to serve a defensive function because it allows to the child to be with the caregiver while avoiding the risk of having his request for comfort denied (avoidant attachment) (Main and Solomon, 1990).

Although these three attachment patterns are different from each other, they are considered to imply the use of "organized" strategies which infants carry out in order to establish a maximum of proximity given the infant's expectations of the caregiver's likely response (Main and Solomon, 1990). In contrast, disorganized attachment has been described as the breakdown of any consistent and organized strategy of emotion regulation during separation and reunion. Disorganized attachment behaviours are considered to reflect profound dysregulation of emotion because the caregiver represents simultaneously the source of fear as well as the only potential haven of safety (Van IJzendoorn et al., 1999). The antecedents of disorganized attachment are associated with specific parental behavioural and psychological problems. In fact, a high percentage of disorganized attachment has been found in maltreated infants (Carlson et al., 1989) but, also, in infants whose caregiver is struggling with unresolved loss of an attachment figure or with other traumatic experiences (Van IJzendoorn, 1995). The development of disorganized attachment has become the focus of intense research interest as it is considered to represent the pattern of attachment most at risk for future psychopathology (Carlson, 1998).

## ATTACHMENT AND DEVELOPMENT

Bowlby had a dynamic view of development on the basis of which not only history and circumstances are important in determining particular outcomes but also the process according to which new experiences can transform established patterns of adaptation and also be interpreted by and in part created by previous history of adaptation (Sroufe, 2005). Consistent with this view, individual differences in attachment are generally considered to be related to outcomes only probabilistically and only in the context of complex developmental systems and processes (Sroufe, 2005). However, the crucial role played by attachment in the initiation of complex processes and its connections with many developmental functions have been suggested in several studies.

The *Minnesota Longitudinal Study* is the main longitudinal study which has investigated the outcomes of different patterns of attachment. This high-risk sample of around 200 subjects was studied over a period of 30 years, measuring different child, family, and context variables through ongoing assessments each and every step of the way (e.g. Carlson, 1998; Sroufe et al, 2005). Specifically, the growth of self-reliance, the capacity for emotional regulation and social competence were investigated through different observational and report measures, employed in multiple contexts and rated by different informants in different times (preschool, middle childhood, adolescence and adulthood). To sum up the main findings, secure infants are more likely to be considered independent, self-confident, ego-resilient as well as with higher social competencies during later development than anxious resistant and avoidant infants. This important study also investigated the role of attachment patterns in predicting later psychopathology: avoidant and resistant patterns were found to be only moderate risks for disturbances, disorganized attachment was shown to be a quite strong predictor of later disturbance. In fact, in adolescence avoidant infants tended to show conduct problem, resistant infants tended to develop anxiety disorders and disorganized infants show dissociative symptomatology. Thus, although attachment security cannot be considered as a guarantee of healthy functioning, it may represent a protective factor with regard to pathology (Sameroff, 2000, p.35); similarly, attachment insecurity can be seen as a risk factor which increases the possibility of having a disorder.

Furthermore, other studies have reported how a history of secure attachment is associated with 1) better performances both in mentalising tasks as the standard false-believe one (Fonagy et al., 1997, Meins et al., 1998) and problem solving tasks (Matas et al., 1978), 2) better regulation of emotional expression in different context such as the Strange Situation (Malatesta et al., 1989) and play (Lutkenhaus et al., 1985) but also a better ability to perceive accurately the expressed emotions of others (Laible and Thompson, 1998), 3) better general cognitive activity and, particularly, language skills (Bretherton et al., 1979; Van IJzendoorn et al., 1995), as well as higher abilities in metacognitive activity (e.g. monitoring and evaluation) (Moss et al., 1993), and 4) better regulation of physiological reactivity in stressful situations (Nachmias et al., 1996; Spangler and Grossman, 1993).

This latter finding is of course highly pertinent to the current discussion and will therefore constitute the topic of the next section.

## THE ROLE OF ATTACHMENT IN COPING WITH STRESS

During the first year of life, caregivers arguably constitute the most powerful resource for children to cope with stress. Sensitive and attentive adult caregivers - who allow their children to express and experience distress and to communicate emotions in ways that can elicit help - appear to be able to buffer the glucorticoid response (Gunnar and Donzella, 2002). In the absence of a history of responsive care toddlers appear to evidence increases in cortisol (Gunnar and Donzella, 2002).

The results of studies that have investigated adrenocortical activation during the Strange Situation are in line with the specificity model described above, as all of them have demonstrated that securely attached infants do not show an altered HPA axis response during separation in the Strange Situation (Hertsgaard et al, 1995; Nachmias et al., 1996; Schieche and Spangler, 2005; Spangler and Grossman, 1993; Spangler and Schieche, 1998). These relatively consistent results makes sense from the point of view of attachment theory, as infants with a secure attachment relationship should feel protected in the presence of the attachment figure and, therefore, should be more able to cope with stress than insecurely attached infants. In other words, attachment security should reflect one aspect of child's coping resources and it could function as a social buffer against less adaptive temperamental dispositions (Gunnar et al., 1989; Nachmias et al., 1996) and new developmental challenges, such as adaptation to child care (Ahnert et al., 2004) or challenging problem-solving tasks (Schieche and Spangler, 2005).

On the other hand, the available evidence regarding insecure attachment relationships as a risk factor for a heightened HPA axis response to separation is mixed and not unequivocal. There is some evidence that disorganized infants exhibit higher cortisol concentrations after the Strange Situation than infants in the traditional (ABC) classifications (Hertsgaard et al., 1995; Spangler and Grossman, 1993). This finding is again in line with the coping model, which assumes that the adrenocortical system would be activated only if adequate behavioural strategies cannot be applied. In fact, as disorganized attachment is thought to represent the breakdown of any coherent strategy for obtaining proximity to the caregiver (Main and Solomon, 1990), the higher cortisol levels found in disorganized infants makes good theoretical sense. However, a recent study did not replicate this finding (Spangler and Schieche, 1998).

Furthermore, studies differ in the extent to which the other insecure categories of attachment show greater increases in cortisol response during the Strange Situation. In particular, avoidant attachment was found to be related to adrenocortical reactivity in Spangler and Grossman (1993) study but not in other studies (Gunnar et al., 1989, Hertsgaard et al., 1995; Spangler and Schieche, 1998); similarly, as far as anxious-resistant attachment is concerned, some studies have reported an association between this attachment pattern and higher cortisol levels (Spangler and Schieche, 1998; Spangler and Grossman, 1993) whilst others did not (Gunnar et al., 1989; Hertsgaard et al., 1995).

According to Spangler and Schieche (1998), there are methodological and psychological explanations which should be kept in mind for better understanding the inconsistency of these results. As far as methodological issues are concerned, the time schedule of cortisol assessment (in term of missing baseline values or early post-assessment of cortisol) or the small number of participants per attachment groups could have contributed to generate not univocal results among studies. Most of the studies reviewed above had small sample sizes and varied the timing of cortisol assessments following the Strange Situation. Possible psychological explanations may also be relevant. For example, it may be that additional, unmeasured, behavioural competencies (such as a low behavioural inhibition) may contribute to psychophysiological regulation in stressful situations and that failure to measure these creates variations in study results dependent on sample variations in these behavioural competences.

Similarly, studies which have tried to examine the role played by attachment patterns in the activation of the sympathetic and parasympathetic systems in response to stress have not generated unequivocal findings.
Sroufe and Waters (1997) found that avoidant infants had accelerated heart rate (HR) both during the separation from the mother and her return in the Strange Situation; this result was confirmed by another study some years later (Spangler and Grossman , 1993) which revealed increased cardiac activity in avoidant and disorganized infants in comparison with secure infants during the Strange Situation. Moreover, the only study which has examined the role played by attachment in explaining individual

differences in alpha amylase levels, as a marker of the sympathetic activity, during the Strange Situation, found higher alpha amylase levels in avoidant infants in comparison to secure infants (Hill et al., 2006). However, a recent study carried out by Zelenko et al. (2005) has shown no associations between individual differences in attachment relationship and HR changes during the separations and reunions with mother in a small sample composed by adolescent mothers and their 1-year-old infants.

Other studies have investigated not only sympathetic but also parasympathetic activity in children with different quality of attachment relationship. Specifically, Stevenson-Hinde and Marshall (1999) measured heart period (HP) and respiratory sinus arrhythmia (RSA) during a modified Strange Situation procedure in preschool children, who were also evaluated for behavioural inhibition by both maternal reports and interviewer's observations. The authors found that HP significantly increased on reunion, but not in high inhibited and insecure children; similarly, RSA increased during the reunion, but not in the group of high inhibited children. Recently, Oosterman and Schuengel (2007) measured changes in HR, RSA and pre-ejection period (PEP) during a separation-reunion procedure in a sample of 3- to 6-year-old children. They reported a significant decrease of RSA over the course of the procedure, as well as on the separation from the mother. However no PEP effects were found thus revealing, in both secure and insecure children, a lack of activation of the sympathetic system.

To sum up, although the quality of attachment is likely to be involved in explaining individual differences in physiological stress response, further research is needed to better understand the interplay between attachment behaviour and the activation of the two stress-related biological systems, taking also into account the role played by constitutional factors. In fact, along with the investigation of temperamental traits which may regulate psychophysiological response in stressful situations, genetic factors related to HPA and SAM activity could account for important components of variance in the stress response.

## II.B) THE EFFECTS OF TRAUMA AND EXTREME PARENTING BEHAVIOURS ON STRESS RESPONSITIVITY

This section of the chapter examines the role played by inadequate parental care, trauma and child maltreatment as important environmental factors involved in physiological stress response. However, they will not be empirically investigated in the current study.

## THE EFFECTS OF PARENTAL CARE ON PHYSIOLOGICAL RESPONSE TO STRESS

Whereas there is a relatively large number of studies which have investigated the impact of child maltreatment on physiological response to stress, very few studies have examined stress-related biological systems in children who have experienced other forms of inadequate parental care such as maternal emotional unavailability or more subtle forms of maltreatment, such as spanking.

Emotion dysregulation can develop from brief or more prolonged separations from the mother as well as from the more disturbing effects of her emotional unavailability, such as occurs when she is depressed. Field (1994) reported how separations from the mother due to her hospitalization or to her conference trips affected the infants' play behaviours and sleep patterns. Comparisons between hospitalizations and conference trips, however, suggested that the infants' behaviours were more negatively affected by the hospitalizations than the conference trips. As the hospitalization of the mother was related to the birth of another baby, it is very likely that the infants perceived their mothers as emotionally unavailable after the arrival of the new sibling. Furthermore, the author showed how the most extreme form of emotional unavailability, mother's depression, had the most negative effects on child's emotion dysregulation. Changes in physiology (heart rate, vagal tone, and cortisol levels), in play behaviour, affect, activity level, and sleep organization as well as other regulating functions such as eating and toileting, and even in the immune system persist for the duration of the mother's depression.

Recently, Bugental and her colleagues (2003) investigated the effects of nonabusive corporal punishment and social-emotional unavailability on hormonal reactivity to stress in a sample of 44 infants and their mothers. Infants who received frequent corporal punishment (e.g. spanking) showed high hormonal reactivity in response to the Strange Situation procedure. In addition, infants who experienced frequent emotional withdrawal by their mothers (either as a result of maternal depression, or mother's strategic use of withdrawal as a control tactic) showed elevated baseline levels of cortisol.

In conclusion, intentionally or unintentionally maternal emotional unavailability as well as inadequate educational tactics (frequent corporal punishment) are likely to produce significant changes in physiological responses of children to stress, thus affecting caregivers' ability to act as social buffer. Further investigations are needed to determine how long the effects of such early dysregulation endure, how they affect the infant's long-term development, how their effect differs across individuals and across development, and whether they can be modified by early intervention.

## THE EFFECTS OF TRAUMA ON PHYSIOLOGICAL RESPONSE TO STRESS

Researchers who have investigated how trauma experiences can impact on biological stress-related systems have largely focused on the outcomes of the post-traumatic-stress disorder (PTSD). PTSD is characterized by a constellation of symptoms which occur following exposure to an extremely stressful or traumatic event, such as exposure to combat, life threatening natural disasters, being held hostage, train crashes, rape, or physical abuse. Symptoms of PTSD include flashbacks, nightmares, feeling worse with reminders of the trauma, sleep disturbance, avoidance of the trauma, physiological arousal, exaggerated startle response, guilt, emotional numbing, and feeling cut off from other people. The interest in the relationship between physiological reactivity to stress and PTSD is partially related to the observation that there is a similarity between behavioural states associated with the activation of the sympathetic system and those associated with anxiety and fear. For example, one of the most replicated findings in PTSD is related to increases in heart rate and blood pressure with exposure to traumatic reminders and might also be a result of abnormal noradrenergic function. The fact that increased release of catecholamines occurs during exposure to stress, in addition to the similarity between many symptoms of PTSD and behavioural effects of catecholamine administration, has led to the idea that alterations in catecholaminergic function may be associated with PTSD. Evidence based on clinical studies supports this idea as well as abnormalities of the HPA axis functioning (for a review see Bremner et al., 1996; Yehuda, 2002). Recently, some studies have reported alterations of both SNS and HPA axis soon after a traumatic event thus suggesting that acute biological responses may serve as risk or resilience factors for the development of PTSD (Delahanty and Nugent, 2006; Yehuda, 2002). Specifically, the few studies that have investigated early biological predictors of PTSD have found an association between the initial sympathetic hyperarousal and an increased risk for PTSD in adults and children. However, the relationship between initial cortisol levels and PTSD symptoms has differed in adults and children (Delahanty and Nugent, 2006). In contrast with adults, traumatized children respond to the event with elevated central corticotrophin-releasing hormone (CRH) and subsequent hypersecretion of cortisol. However, it is possible that over time prolonged elevations of central CRH and hypersecretion of cortisol are likely to disrupt normal HPA axis functioning, ultimately resulting in enhanced negative feedback inhibition of the pituitary and eventually leading to lower basal cortisol levels. Longitudinal studies on child trauma victims who can be at higher risk for future traumatic experiences will contribute to better understand contradictory findings and advance the theoretical understanding of PTSD.

Studies on the biological responses to child maltreatment, which is very likely to represent the most frequent and severe trauma a child can go through, will be reviewed in the next paragraph.

## THE EFFECTS OF CHILD MALTREATMENT ON PHYSIOLOGICAL RESPONSE TO STRESS

Child maltreatment is one of the greatest failure of the caregiving environment to provide most of the expectable experiences that are necessary for promoting adequate developmental processes (Cicchetti and Lynch, 1995). In contrast to what is expected in response to an average expectable environment, the ecological, social, biological and psychological conditions associated with child maltreatment are very likely to lead to a failure and disruption in the successful resolution of main stage-salient issues, thus increasing the probability of developing maladaptation and psychopathology (Cicchetti, 1989; Cicchetti and Lynch, 1995; Cicchetti and Toth, 1995).

Although there is a larger number of studies which have investigated psychological consequences and correlates of maltreatment, the interest of researchers on the impact that abuse has on biological functioning of the individual is increasingly growing in these last years (e.g. Brenmer et al., 1997; De Bellis et al., 1999; Cicchetti and Rogosh, 2001a; Cicchetti and Rogosh, 2001b; Hart et al., 1995, 1996; Ito et al., 1998; Pollak et al., 1997). In the following paragraphs the literature on the effects of child maltreatment on HPA and SNS functioning will be briefly reviewed.

## HPA axis response in maltreated children

Several investigations indicate that there is altered HPA axis functioning in maltreated children: both elevated and suppressed activity of the HPA axis system has been associated with childhood abuse (Gunnar and Donzella, 2002). This is likely to reproduce the complex nature of maltreatment and its association with a variety of different emotional and behavioural sequelae (Cicchetti and Lynch, 1995). In fact, different factors such as child's age when the maltreatment occurred, type of maltreatment, type of psychopathology displayed, subsequent exposure to stressors, and parental responsiveness contribute to influence the degree and patterning of HPA disturbance, thus partially explaining discrepancies in findings (Van Voorhees and Scarpa, 2004).

**Characteristics of abuse**. The type of maltreatment may play a role in determining differences in cortisol responsitivity. Morning and evening cortisol concentrations were found to be related to the severity of abuse in a sample of 167 school-aged maltreated children and 204 demographically comparable low-income non maltreated boys and girls (Cicchetti and Rogosh, 2001a). Particularly, maltreated children who were both physically and sexually abused (as well as neglected or emotionally maltreated) had high elevations in morning cortisol levels, and a subgroup of them were at high levels of cortisol in both the morning and the afternoon. The developmental timing of maltreatment and the severity of sexual abuse were also measured in order to ascertain whether other characteristics of maltreatment could provide further insight: whereas the developmental timing of maltreatment did not appear to account for the cortisol elevations, the severity of sexual abuse did. On the other hand, children who had experienced physical abuse showed evidence of a trend toward lower morning cortisol levels, and a subgroup of them also evidenced a rise in afternoon cortisol rather than the expected decrease. Last, the patterns of cortisol regulation of both the neglected and the emotionally maltreated groups of children did not differ from the group of not maltreated children. The authors of this interesting study (Cicchetti and Rogosh, 2001a) conclude that the hypercortisolism and hypocortisolism, manifested by the sexually and abused group and the physically abused group respectively, might lead to enduring neurobiological compromise.

**Type of psychopathology.** There is empirical evidence that child maltreatment is a strong risk factor for the development and persistence of mental disorders such as major depressive disorder and post-traumatic stress disorder. Recently, several studies have investigated the neuroendocrine functioning of maltreated children who have been diagnosed with psychopathological disorders (De Bellis et al., 1999; Cicchetti and Rogosh, 2001b; Hart et al., 1995, 1996; Kaufman, 1991, 1997; Shea et al., 2004; Van Voorhees and Scarpa, 2004). These kinds of studies are particularly interesting as they can provide insight into how the experience of maltreatment affects the typical patterns of HPA axis functioning observed in individuals with mental disorders but who did not experience maltreatment in their life.

Kaufman (1991) first reported an increase rather than the normal decrease in cortisol levels from morning to afternoon in a sample of maltreated children who had a diagnosis of major depression. This finding was replicated and extended by Hart and colleagues (1996) who investigated the effects of maltreatment on adrenocortical responsitivity in a large sample of low socioeconomic status school-age maltreated and nonmaltreated children. The authors found that morning cortisol levels were lower in depressed maltreated children than in non depressed maltreated children.

Furthermore, some of depressed maltreated children showed an atypical increase in cortisol levels from the morning to the afternoon. In a follow-up to her 1991 study, Kaufman and colleagues examined HPA axis functioning by administering the CRF stimulation test to depressed maltreated, depressed nonmaltreated, and normal control children and found two different ACTH responses to CRF administration in the depressed maltreated group. Specifically, they found an increased ACTH secretion post- CRH infusion only in the depressed maltreated children who were experiencing ongoing chronic adversity whereas no increase was evident in the depressed maltreated children who experienced maltreatment in the past. De Bellis and colleagues (1999) investigated cortisol regulation in a small group of maltreated prepubertal children who had a diagnosis of posttraumatic stress disorder (PTSD) and matched controls: maltreated children with PTSD excreted significantly greater 24-h urinary free cortisol (UFC) than nonmaltreated children. PTSD symptoms and the duration of abuse were also found to positively correlate with UFC concentrations. Finally, Cicchetti and Rogosh (2001b) looked into cortisol regulation in their large sample of maltreated and notmaltreated school aged children, previously cited. They reported that maltreated children with clinical-level internalizing problems were distinguished by higher morning, afternoon and average daily cortisol levels. Thus, there is empirical evidence that abused children with internalizing symptoms and disorders manifest dysregulation of the HPA axis functioning.

In contrast with the growing number of studies which have investigated adrenocortical reactivity in maltreated children who suffered internalizing symptoms and disorders, there is a paucity of research on the neuroendocrine functioning of maltreated children with clinical levels of externalizing problems. In their study, Hart and colleagues (1996) reported that children with clinical levels of externalizing problems, regardless of maltreatment, showed lower cortisol levels averaged across the day. Cicchetti and Rogosh (2001b) found that the non maltreated boys with clinical level externalizing problems had the lowest average daily cortisol levels thus apparently limiting the effect of maltreatment in relation to externalizing problems and to cortisol secretion. Furthermore, maltreated children with comorbid externalizing and internalizing problems were more likely not to show the expected diurnal decrease in cortisol levels.

To sum up, there is not a consistent pattern of cortisol regulation for all maltreated children across studies. These findings could be considered to indicate that the brains of all children are not uniformly affected by the experience of abuse.

**Parental responsiveness.** The quality of the child-mother attachment relationship is seen as another significant factor which should be taken into account in order to explain the individual differences in glucorticoid responses observed in maltreated children (Van Voorhees and Scarpa, 2004). Particularly, disorganized attachment could play a significant role, as there is a high rate of this pattern of attachment in samples of maltreated children (Van IJzendoorn et al., 1999). Maltreating caregivers, in fact, are thought to be one of the causes of disorganized attachment of their infants because they place their children in the paradoxical situation of "fright without solution", representing contemporaneously the only source of comfort but also the source of threat to their security (Hesse and Main, 2000). While several investigations have been conducted that indicate that there is altered HPA axis functioning in maltreated children, there is no study in which cortisol concentrations have been assessed in relation to the Strange Situation procedure in abused infants. However, there is some evidence that disorganized children exhibit higher cortisol concentrations than infants in the traditional (ABC) classifications (Hertsgaard et al., 1995; Spangler and Grossman, 1993). In the light of this, the investigation of the role played by attachment as a possible moderating variable of HPA axis functioning seems to be an intriguing issue to address.

## SNS Functioning in maltreated children

Most of the studies which have looked into the impact of maltreatment on the sympathetic nervous system have been carried out in samples of children with PTSD following to maltreatment. Different studies have investigated urinary catecholamine concentrations as they reflect plasma and peripheral SNS activity, tonic stimulation of the adrenal medulla, and metabolic breakdown of catecholamines. In a series of studies, De Bellis and colleagues reported a higher baseline activity of the catecholamine system in maltreated children (De Bellis et al., 1994; De Bellis and Putman, 1994; De Bellis et al., 1999). In an initial pilot study, these authors (De

Bellis et al., 1994) examined urinary catecholamine excretion in a self-selected sample of sexually abused and demographically matched control girls recruited from a prospective longitudinal study. The sample of abused girls were found to secrete significantly greater amount of homovanillic acid, a metabolite of dopamine, than the comparison group, thus indicating higher catecholamine activity. In their further study, De Bellis and colleagues (1999) measured urinary catecholamine excretion in a sample of prepubertal children with PTSD secondary to past child maltreatment, compared to non-traumatized children with over anxious disorder (OAD) and healthy controls. The authors found that maltreated children with PTSD excreted significantly greater concentrations of urinary dopamine and norepinephrine over 24 hours than OAD and controls children, and greater concentrations of epinephrine than children with OAD. Moreover, urinary catecholamine concentrations showed positive correlations with duration of the PTSD trauma and severity of PTSD symptoms. Perry (1994) has reported decreased platelet adrenergic receptors in a small group of children with PTSD following serious abuse, a finding suggesting downregulation of the peripheral adrenergic receptors in response to higher levels of circulating catecholamines. Furthermore, an overactive sympathetic nervous system in these children were also suggested by increased resting heart rate, and abnormal return of hearth rate to baseline levels after an orthostatic challenge. These findings are presumed to indicate an enduring stress response and support a similarity to the psychobiology of post-traumatic stress disorder in adults.

Last, Galvin and colleagues (1991) found an association with reduced levels of plasma dopamine beta hydroxylase (D$\beta$H) in a sample of psychiatrically hospitalised boys who had experienced significant maltreatment early in their life. This enzyme is involved in the conversion of dopamine to norepinephrine. The reduced blood level of the enzyme, which is correlated with D$\beta$H level in the cerebrospinal fluid (CSF), is believed to be long-lasting. Galvin and colleagues, postulate that the early neglect and abuse which these boys suffered led to an overstimulation of the noradrenergic system due to the stress response with enzyme induction. Subsequent reactive repression of enzime activity leads to the findings of lowered D$\beta$H level.

To sum up, all studies here reviewed support a hyperactivation of the SNS in maltreated children although the limited sample size of some samples, the different assessments of maltreatment, the different clinical populations and methods employed make difficult the generalizability of the findings.

In the following section, the role played by child constitutional factors in explaining individual differences in physiological response to stress will be reviewed.

## PART III. THE EFFECTS OF CONSTITUTIONAL FACTORS ON STRESS RESPONSITIVITY: THE ROLE OF GENETICS AND TEMPERAMENT

Individual differences in the stress response and the predisposition for stress-related pathology may be the product of both environmental and constitutional determinants. Studies which have addressed the extent to which genetic factors contribute to individual differences in response to stress will be reviewed in this section. In addition, this section will provide a synthesis of the studies that have investigated the role of genetic polymorphisms in the development of attachment disorganization, as this has been associated with abnormal physiological functioning. Last, empirical studies on the impact of child temperament and its interrelationships with social context and attachment on the functioning of stress-related systems will conclude the chapter.

### III. A) THE ROLE OF GENES IN EXPLAINING INDIVIDUAL DIFFERENCES IN STRESS-RELATED SYSTEMS

There is a large consensus that genetic factors are responsible for part of the individual variation in emotional reactivity and neuroendocrine stress responses, as shown by family and twin studies in humans, and by the study of inbred strains and selection experiments in animals. Although interindividual variability in sympathoadrenal responses to stress is recognized, the determinants of the variability, and in particular the role of genetic factors, are poorly understood. Moreover, the few studies of genetic influences on SAM functioning have involved animals rather than humans, while human work has focused on genetic vulnerability to HPA axis dysregulation. Consequently, this review will mainly focus on genetic determinants of the HPA axis functioning whereas a lesser attention will be given to those of sympathoadrenal system.

**GENETICS AND STRESS RESPONSE**

Researchers who are interested in understanding how genetic factors can contribute to explain stress-related systems functioning in humans have focused on twin studies and association studies with polymorphisms in some "candidate" genes.

## Twin studies

Few twin studies have investigated the influence of genetic factors on hormones associated with HPA axis functioning: while some of these have looked into baseline HPA axis activity, others have focused on the stimulated activity of this system.

Although most independent studies on baseline HPA axis measures in twins suggest a moderate contribution of genetic factors (Maxwell et al., 1969; Meikle et al., 1988; Wüst et al., 2000), stronger support for a moderate-high heritability is provided by a reanalysis of the baseline cortisol levels reported in five twin studies using genetic model fitting and structural equation modeling procedures (Bartels et al., 2003a). Furthermore, a recent report on 180 twin pairs shows that similar heritabilities can be found in 12-year-old children (Bartels et al., 2003b). Specifically, a significant genetic contribution was found to the variation of basal cortisol levels in the morning and afternoon samples, but not in the evening sample.

Studies on the heritability of stimulated HPA axis activity employed rather diverse experimental procedures and yielded inconsistent findings regarding estimates of heritability (Wüst et al., 2004). In the first study on possible genetic influences on variability in adrenocortical reactivity, no significant twin concordances were found for cortisol responses to the administration of dextroamphetamine in a small sample of MZ and DZ twin pairs (Nurnberg et al., 1982). Kirschbaum and colleagues (1993) reported differing heritabilities for stimulated cortisol levels, depending on the nature of the stimulus. Specifically, in this study the free cortisol response to a challenge with human corticotropin releasing hormone (h-CRH), ergometry, and the Trier Social Stress Test were assessed in a small sample of MZ and DZ. Genetic factors seemed to influence salivary cortisol peak levels after h-CRH administration ($h^2$ = .84), but were less important after TSST exposure ($h^2$ = .32) and were not detectable after stimulation with ergometry.

In a larger sample of twins (Inglis et al., 1999), an influence of genetic factors was detectable for variation in 11-deoxycortisol ($h^2$ = .54), 11-deoxycorticosterone ($h^2$ = .40), and to a lesser extent for corticosterone ($h^2$ = .28). However, no heritable component could be detected for stimulated levels of cortisol, aldosterone, and 11β-hydroxylase. Froehlich et al. (2000) investigated cortisol, ACTH, β-endorphin, and prolactin responses to stimulation with ethanol in 51 MZ and 37 DZ pairs: a genetic influence for variation in β-endorphin was found but no signs of heritability for cortisol, ACTH, and prolactin were detectable. Finally, a recent study (Federenko et al., 2004) has examined the effects of context on the heritability of psychoendocrine stress responses in 58 male twin pairs. Participants were exposed to a psychosocial stressor (Trier Social Stress Test) three times at weekly intervals, and their salivary and total cortisol, ACTH, and heart rate responses were assessed. Modest heritabilities were observed for all measures at the first stress exposure (all h2 < 0.33), but heritability estimates increased substantially with repetition of the stressor (T3: all h2 > 0.97) thus suggesting low heritabilities in a new, anxiety-evoking context and high heritabilities in a familiar, low-anxiety context. These results strongly suggest that contrary to previous results, genetic factors do contribute to variability in stimulated cortisol and ACTH responses.

## "Candidate genes" of the stress response

Clearly, HPA axis and SAM system functioning as well as individual vulnerability for stress systems-related clinical states are complex phenotypes which are influenced by many genes and environmental conditions. Amongst "candidate" genes supposed to play a significant role in determining individual differences in response to stress, those implicated in the functioning of neurotransmitters, such as norepinephrine, dopamine and serotonin, are of particular interest given their functions within stress-related brain systems.

In the present literature review, the serotonin transporter gene (5- HTTLPR), the Catechol-O-Methyltransferase gene (COMT), and the dopamine D 4 receptor gene (DRD4) as "candidate" genes in stress response will be described, jointly with a brief description of the Glucorticoid Receptor Gene in the light of recent data (Wüst et al., 2004) reporting its significant contribution to HPA axis response to a psychological stressor.

**The serotonin transporter gene (5-HTT).** One of the neurotransmitters implicated in LHPA axis and behavioural aberrations observed in animals exposed to early life stress is serotonin. During the early postnatal period, serotonin plays a crucial role in the development of the central nervous system (reviewed in Lauder 1983), and serotonin neurotransmission is involved in both activation and feedback control of the LHPA axis (Cassan and D'mello, 2001; Feldman and Weidenfeld, 1998; Lowry, 2002; Weidenfeld et al., 2002). Moreover, studies have shown a direct effect of serotonin on hippocampal type II glucocorticoid receptors (Mitchell et al., 1990a, 1990b), which are critically involved in fast-feedback termination of LHPA axis activity (Keller-Wood and Dallman, 1985; Young and Vazquez, 1996).

Because serotonin's action in the synapse is terminated by reuptake, the serotonin transporter is critical in regulating its function. Several studies suggest that naturally occurring variants in the serotonin transporter gene could potentially increase an individual's susceptibility to allostatic load (Barr et al., 2004). In humans, a common polymorphism involving an insertion/deletion in the promoter region of the serotonin transporter gene (5- HTTLPR) alters in vitro gene transcription (Heils et al., 1997), in vitro transporter activity (Stoltenberg et al., 2002), and in vivo serotonin transporter density (Heinz et al., 2001). Individuals carrying the short (*s*) allele are more likely to demonstrate anxiety-related personality traits, such as neuroticism, harm avoidance, and disagreeableness, than are individuals homozygous for the long (*l*) allele (Costa and McCrae, 1997; Greenberg et al., 2000; Lesch et al., 1996; Mazzanti et al., 1998; Van Gestel et al., 2002). Other studies demonstrate increased activation of the right amygdala in response to fearful stimuli in carriers of the short allele (Hariri et al., 2002). Recently, a study by Caspi et al. (2003) demonstrated the *s* allele to be associated with depression, but only among individuals exposed to major life stressors. Last but not least, a series of elegant studies carried out by Suomi and his colleagues with rhesus monkeys have shown not only that LHPA axis activity is affected by serotonin transporter gene variation, but also that the influence of 5-HTTLPR on hormonal responses is modulated by early experience (e.g. Barr et al., 2004; Suomi, 2006).

**Catechol-O-Methyltransferase Polymorphism (COMT).** The secretion of norepinephrine plays a significant role in regulating the stress response. Therefore, it is possible that genetically determined differences in NE metabolism might influence HPA axis functioning. Catechol-O-methiltransferase (COMT) is an enzyme responsible for cathecolamine catabolism in the brain and other tissues, such as the liver, kidney, and heart. Lachman and colleagues (1996) reported that a common functional polymorphism in the COMT gene, resulting of a G to A mutation that translates into a valine (val) to methionine (met) substitution at codon 158, accounted for a fourfold decrease in enzyme activity. Homozygosity for the met allele is associated with low enzyme activity, homozygosity for the val allele is associated with high activity, and because the alleles are codominant, heterozygosity is associated with intermediate levels of activity. Recently, Oswald and colleagues (2004) investigated individual differences in adrenocorticotropin hormone and cortisol responses in adults with the met/met genotype and adults homozygous or heterozygous for the val allele. In their study, 46 healthy participants were genotyped and underwent a procedure in which adrenocorticotropin hormone and cortisol responses to the opiod receptor antagonist naloxone were measured. Adults with the met/met genotype had greater ACTH and cortisol responses to naloxone than adults who carried the val allele. Therefore, it is suggested that individual differences in cathecolamine metabolism may impact HPA axis function and may play a pharmacogenetic role in response to naloxone. However, additional research is needed to better clarify whether and how the low activity genotype may induce an enhanced HPA axis response to either real life or laboratory-induced stress.

**Dopamine D4 receptor gene (DRD4).** In animal studies, disruptions of the mother-infant relationship have been shown to have long-lasting effects on the mesolimbic dopamine system and the hypothalamic pituitary adrenal axis (Liu et al., 1997; Matthews et al., 1996; Meaney et al., 2002). For example, adult rats that were separated from their mother in early life have fewer dopamine reuptake transporters, most prominently in the ventrial stratum (Meaney et al., 2002). Interestingly, Pruessner et al. (2004) have recently reported a significant association between an anxiety inducing stress task and dopamine release in the ventrial striatum in human subjects. Consequently, genes relevant for the dopaminergic neurotransmission might, to a certain extent, explain individual differences in stress response.

Two variants in the dopamine D4 receptor (DRD4) gene, namely DRD4 gene variable number of tandem repeats (VNTR) and C-521T polymorphisms, have been reported to be associated, alone or combined, with various psychological traits and several behavioural and psychiatric disorders both in adults and in children. The human dopamine D4 receptor (DRD4) was shown to display a large polymorphic variation as a consequence of a 48 basepair variable number tandem repeat (VNTR) sequence, repeated 2-10 fold in the third exon of DRD4 gene (Van Tol et al., 1992). The 7-repeat allele was found to have a lower potency for dopamine-mediated coupling to adenylate cyclase than receptors encoded by the 2- or 4- repeat forms (Asghari et al., 1995). This variant has found to be associated with pathological impulsive behaviour and substance abuse in adults (e.g. Ebstein et al., 1996), as well as with infant maladaptive temperamental traits, Attention Deficit Hyperactivity Disorder (ADHD) and disorganized attachment in children ( e.g. Auerbach et al., 1999; Faraone et al., 2005; Lakatos et al., 2000).

One of the most studied polymorphisms of the DRD4 promoter region is the -521 C/T single nucleotide polymorphism (SNP), a C>T substitution in the promoter region of the same gene. A positive association between novelty seeking and the -521 CC genotype has been reported (Schinka et al., 2002), as well as between the -521 C allele and schizophrenia (Okuyama et al., 1999), although others failed to replicate this latter result (Mitsuyasu et al., 2001). A weak relationship between the -521 C/T SNP and ADHD has also been reported (Lowe et al., 2004). Further, Lakatos et al. (2002) revealed an association between the infant disorganized attachment and the T allele of the -521 C/T SNP but only in combination with the 7-repeat form of the DRD4 exon III polymorphism. A family-based association approach demonstrating a low-rate transmission of the DRD4 7-repeat –521 T haplotype in a large secure group of infants (Gervai et al., 2005) supports this finding, although results to the contrary have also been published (Bakermans-Kranenburg and Van IJzendoorn, 2004).

However, as far as we know, both DRD4 gene polymorphisms have never been investigated in psycho-biological studies on stress response.

**Glucorticoid Receptor Gene Polymorphisms.** The glucorticoid receptor (GR) is a member of the steroid receptor superfamily of ligand-activated transcription factor. It mediates many of the effects of GCs on target tissues via direct binding to hormone-responsive elements in the DNA or via interactions with other transcription factors resulting in a modulation of gene transcription (Reichardt et al., 1998). A cell's response to GCs is predominantly determined by both the steroid level it is exposed to and by its GC sensitivity, i.e. the efficiency of GR-mediated signal transduction (Bamberger et al., 1996). Evidence from recent studies suggests that variants of the GR gene (located on chromosome 5, locus 5q31) that affect a cell's sensitivity for GCs may contribute significantly to the large interindividual variability of HPA activity and GC sensitivity of target tissues in normal, non clinical populations (DeRijk et al., 2002).

The impact of three GR gene polymorphisms, namely the *BclI* restriction fragment length polymorphism (RFLP), the single nucleotide polymorphysm N363S, and the ER22/23EK on HPA responses to psychological stress was recently investigated in a sample of 112 young males by Wüst and colleagues (2004a). Particularly, these GR gene polymorphisms were analysed in relation to cortisol and ACTH responses to pharmacological stimulation and psychosocial stress. The pharmacological stimuli were provided by the ACTH1–24 challenge and dexamethasone suppression tests, whereas the Trier Social Stress Test (TSST) was repeatedly used for the application of acute psychosocial stress. The authors reported a significant impact of GR polymorphisms on cortisol responses to psychosocial stress suggesting that 363S allele is related to increased responses to a psychosocial stress procedure whereas the *BclI* genotype GG seems to be associated with a relative hyporeactivity. Differences across genotypes in response to pharmacological stimulations were less evident. After dexamethasone ingestion, males with 363S showed a trend toward an enhanced cortisol suppression. In conclusion, common polymorphisms of a single gene were proved to have modulating effects on the relation between psychological factors and HPA regulation. However, although the GR gene is undoubtedly a good candidate gene associated with cortisol activity the low prevalence of the risk genotype in general population precludes its investigation in the present sample.

In the next paragraph, genetic studies which have examined the antecedents of disorganized attachment will be reviewed in the light of the theoretical importance given by attachment theory to this attachment pattern in the dysregulation of child's strategies to cope with stress (Main and Solomon, 1990).

## GENETIC STUDIES ON ATTACHMENT DISORGANIZATION

The increasing interest in investigating the weight played by genetic factors on childhood development has also been extended to the child-mother attachment relationship. Although there is no direct correspondence between a gene mutation and the presence of a definite behavioural trait or disorder, the influence of genetic components on physiological and behavioural phenotypic variations cannot be ignored (McGuffin et al., 2001).

Several studies have examined the influence of genetics on infant-parent attachment, focusing on the role played by (shared - non shared) environmental factors and genetic factors in mono- and dizygotic twin populations (Bokhorst et al., 2003; Finkel et al., 1998; Finkel and Matheny, 2000; O'Connor and Croft, 2001; Ricciuti, 1992). Whereas most of them found a negligible effect of genetics on individual differences in attachment (Bokhorst et al., 2003; Ricciuti, 1992; O'Connor and Croft, 2001), a recent molecular genetic study has reported a strong association between disorganized attachment and the presence of a polymorphism of the dopamine D4 receptor (DRD4) gene in a low-risk population (Lakatos et al., 2000). The same research group found that this association was enhanced in the presence of another polymorphism on the upstream regulatory part of the same gene (Lakatos et al., 2002). Specifically, the interaction between the structural 48-bp repeat polymorphism and the –521 C/T promoter polymorphism made the odds ratio for disorganized attachment increase tenfold. The authors' conclusions about their findings are related to the role played by the mesolimbic dopamine system in determining the motivational value of a stimulus. According to Lakatos and colleagues (2000), indeed, *a less sensitive D4 dopamine receptor in this area could reduce the motivational value of the returning mother and prevent the infant from organizing an appropriate response.*

Another repeat polymorphism which has been studied by Lakatos and colleagues (2003) is the 22 bp VNTR in the promoter region of the serotonin transporter gene (5HTTLPR), the short allele of which was found to enhance avoidant like behaviour (by an interaction with the DRD4 repeat variant) (Auerbach et al, 2001; Ebstein et al, 1998) and risk for depression in childhood (Nobile et al., 2004). However, no effect of the 5-HTT gene on the formation of disorganized attachment was found. Interestingly, a study which has adopted a behavioural genetic as well as a molecular genetic approach in order to verify if Hungarian group findings could be replicated has recently been published (Bakermans-Kranenburg and Van IJzendoorn, 2004). Bakermans-Kranenburg and Van IJzendoorn (2004) have looked into the association between disorganized attachment and the two polymorphisms of the DRD4 gene in a low-risk sample of seventy-six mono – and dyzgotic twins. They found no association of disorganized attachment with the 7-repeat DRD4 allele, the –521C/T genotype or the interaction of both polymorphisms. They concluded that the empirical evidence to date support primarily environmental influences on attachment rather than genetic factors as antecedents for disorganization of attachment in infancy.

The difference in the results of the two studies that have investigated DRD4 in relation to attachment disorganization is difficult to explain, but given the importance of dysregulation in behavioural coping implicated in disorganized attachment it may be that genetic effects are more prominent in higher-risk environments. Recent studies have been focusing on the investigation of the interplay between adverse environmental conditions and genetics in the development of disorganized attachment (Gervai et al., in press; Van IJzendoorn and Bakermans-Kranenburg, 2006).

## III. B) THE ROLE OF TEMPERAMENT IN EXPLAINING INDIVIDUAL DIFFERENCES IN STRESS-RELATED SYSTEMS

Temperament is viewed as a behavioural style, a biological or bio-behavioural set of traits, or as embedded in socialization contexts. In this brief review, it will be considered in relation to "the psychobiological study of individual differences in basic behavioural response styles or dispositional traits" (Nigg, 2006). Basic behavioural traits refer to higher order patterns of individual differences in reactivity to immediate incentive contexts, as well as their mutual regulation, and lower order constituent traits that make up these higher order traits; furthermore, they can be conceptually and empirically related to distinct neural networks and, to some extent, partially distinct peripheral nervous system (PNS) probes. Temperament is suggested to be a multi-level enterprise, such that relevance is found in the behavioural level (e.g., behavioural inhibition in unfamiliar contexts), the psychological level (e.g., anxiety), the neural level (e.g., activity/reactivity of limbic networks), the physiological level (e.g., autonomic arousal), and the genetic level (e.g., role of serotonin-modulating genes) and that looking for interactions and mediations across these levels is a key goal (see Canli, 2004; Calkins and Fox, 2002; Cicchetti and Dawson, 2002; Kagan et al., 2002; Zuckerman, 2001).

Significant normative changes in behavioural expressions of temperament happen during the early preschool years (see Emde and Hewitt, 2001) thus evidencing that different aspects of temperament are salient at different points in development. For example, negative affect may be more apparent in young babies than in older children, and activity level characterizes young children but does not tend to emerge as a separate trait in older children and adults (Putman et al, 2001). Moreover, temperament-based behaviour is apparent only in relevant incentive contexts (Wachs and Kohnstamm, 2001).

## Temperamental traits associated with physiological reactivity

Behavioural inhibition is probably the most investigated temperamental trait by studies which have examined the interrelation between temperament and physiological response to stress in childhood (e.g. Gunnar et al., 1989; Kagan et al., 1987; Schmidt et al., 1997; Wilson et al., 2003). Behavioural inhibition reflects a tendency to display fear and wariness in response to novel stimuli. Behaviourally inhibited children display long latencies to approach novel stimuli, exhibit a high frequency of negative effect, and remain in close proximity to their mothers in response to the presentation of novel stimuli. Children who display this pattern of behaviour towards novel social stimuli have been characterized as shy and timid. Researchers have suggested that consistently wary and inhibited infants and children show a particular physiological profile comprising a high and stable heart rate, elevated cortisol, and right frontal EEG activation (e.g., Calkins et al., 1996; Fox et al., 1995; Kagan et al., 1987; Marshall and Stevenson-Hinde, 1998). However, as the HPA system is perhaps the most extensively studied of all stress-responsive physiological systems, the attention will be focused on studies which have investigated this system and, particularly, the cortisol response.

Kagan et al. (1987) noted that children who were behaviourally inhibited at 21 months displayed higher morning and laboratory cortisol levels at 5 ½ years compared with their uninhibited counterparts. They also found that children who were contemporaneously inhibited displayed high morning cortisol at 5 ½ years. Still others have noted elevated adrenocortical activity in human infants whose temperamental dispositions may be predictive of behavioural inhibition in early childhood. Gunnar and her colleagues (1989), for example, reported that human infants who remained temperamentally distressed from 9 to 13 months of age displayed increases in adrenocortical activity during laboratory play vignettes. Schmidt et al. (1997) examined social behaviours, maternal report of temperament, salivary cortisol, and baseline startle response in 4-year-old children. They found that infants who displayed a high frequency of motor activity and negative affect at 4 months of age were likely to be rated by their mothers as more shy at age 4 compared with other children. Furthermore, 4-year-old children who were extremely wary of social novelty during peer play exhibited a) relatively higher morning salivary cortisol, b) were more likely to be rated contemporaneously shy by their mothers, and c) were more likely to have been behaviourally inhibited at 14 months of age compared with other children. However, no significant relations were found between baseline startle and morning salivary cortisol and measures of shyness at age 4. In a recent study, Wilson and colleagues (2003) investigated the relationships among physiologic and behavioural response to a stressor (immunizations) and temperament in the first year of life. The sample was composed by infants who were assessed at 2 months and 4 months of age during routine clinic visits. Saliva samples were collected before and 20 minutes after immunizations, two behavioural responses (latency to dampen distress and latency to calm) were coded from videotapes filmed for 90 seconds after immunizations, and a questionnaire on temperament was completed by parent for each assessment time. Two-month-old infants with higher levels of cortisol required more time to calm after immunization. Cortisol changes in this study were related to the temperament characteristics of intensity, rhythmicity, approach-withdrawal, and distractibility. More intense children showed greater physiologic arousal at both 2 and 4 months. At 4 months, infants with more withdrawing temperaments had higher cortisol levels. Thus, the temperament correlates of physiologic reactivity (approach-withdrawal and intensity) indeed related to physiologic arousal.

Although these results support the conclusion that infant temperament plays an important role in infants' responses to stress (Lewis and Ramsay, 2003), other studies are at odds with this notion. In fact, high cortisol levels were found in dominant, assertive, and socially competent children during the preschool and early school years (Granger et al., 1994; Hart et al., 1995; Montagner et al., 1978; Tennes and Kreye, 1985).

Some authors suggested that temperament-cortisol associations could be better understood taking also into account other moderating variables. The role played by social context and child-mother attachment relationship as possible factors which needed to be considered in order to better understand the relationships between child temperamental traits and physiological reactivity to stress will be considered in the next paragraphs.

## The influence of social context in explaining the role played by temperament in physiological response to stress

Many studies of stress-sensitive physiological systems have focused on individual differences in temperament rather than associations with qualities of children's social relationships. This focus partly reflects interest in identifying behavioural dispositions associated with stress vulnerability (Boyce and Jemerin, 1990). However, stress reactivity may vary not only across individual but, for an individual, it may vary across different situations (Nicolson, 1992). Specifically, relations between shy, inhibited temperament and cortisol could, at times, be masked by other social factors such as those operating in peer groups that might influence the activity of this neuroendocrine system.

The work of Gunnar and her group is one of the most extended and exhaustive in this field. In their 1997 study (Gunnar et al., 1997), the relations among temperament, social competence, and levels of salivary cortisol were investigated in two studies of preschool children. Cortisol daily for the initial weeks of the school year and for several weeks later in the year were sampled in both studies. The authors found that median cortisol was modestly stable across periods, but cortisol reactivity, measured as the difference between the 75$^{th}$ and 50$^{th}$ percentile, was not. Outgoing, competent, and well liked by their peers children showed high cortisol reactivity (75th minus 50th percentile) during the initial weeks of the school but low-to-normal cortisol reactivity during the weeks later. On the contrary, children who changed from low/normal to high cortisol reactivity and those who maintained high cortisol reactivity from the beginning to the later weeks were affectively negative and solitary. Last, children who showed high median cortisol several weeks after the formation period or over both periods scored lower on a measure of attentional and inhibitory control. In another study of the same research group (de Haan et al., 1998) morning cortisol levels were sampled from twenty-four 2-year-olds at home, during the first week of school, during weeks 6-9 of school, and during the first week following a month-long break. Behavioural characteristics of the toddlers were assessed by classroom observation, teacher and parent reports. The authors found no general increase in cortisol during the first days of preschool compared to their levels at home or later school levels. However, significant individual differences emerged in relation to the psychosocial contexts: specifically, cortisol levels at home were correlated with more shy, anxious and internalizing behaviours whereas the response to starting school was correlated with more assertive, angry, and aggressive behaviour. Thus, children who show high cortisol levels in one context, may have low cortisol levels in other, even similar contexts (e.g. early and later days of school). Recently, Gunnar and her colleagues (Gunnar et al., 2003) tested a model on direct and indirect pathways between cortisol and temperament in social contexts by assessing sociometric measures of peer rejection, salivary cortisol, and teacher reports of temperament in 82 preschool children. Children classified as peer rejected exhibited higher cortisol levels. Specifically, elevated cortisol levels were found in children who were both exuberant and unable to inhibit or control negative impulses through a pathway mediated by aggressive interactions with peers and peer rejection.

In their conclusion, the authors state that the associations between shy, inhibited temperament and cortisol may be masked when children are assessed in peer settings by other qualities of children's behaviour that create socially stressful interactions with others.

To sum up, together these results suggest that relations among temperament, social competence, and neuroendocrine reactivity reflect both individual and contextual differences.

## The influence of child-mother attachment relationship in explaining the role played by temperament in physiological response to stress

The physiological impact of inhibited temperament cannot adequately be understood without an appreciation of the coping resources and coping behaviours available to the child. As already described in this chapter, the security of the child-mother attachment relationship is considered to be a significant coping resource for the child against stress.

The role of the infant attachment in moderating the relations between behavioural inhibition and changes in salivary cortisol levels in response to stressful circumstances was first investigated by Nachmias and colleagues (1996). The authors assessed changes in cortisol levels after the strange situation and an observational procedure devised for measuring toddler inhibition of approach to novel events; toddler coping behaviours and maternal behaviours to help toddlers manage novel events were also investigated. They found that insecure, higher-inhibition infants had higher postsession cortisol levels than secure, higher inhibition children. Furthermore, mothers of inhibited and insecurely attached toddlers were likely to interfere with their children's coping efforts to manage novel events. Spangler and Schieche (1998) investigated quality of attachment, emotional expression and adrenocortical reactivity after the strange situation procedure in a large sample of 126 infants and found that adrenocortical reactivity was most prominent in insecure infants with high behavioural inhibition. Burgess and colleagues (2003) examined attachment classification at 14 months of age, behavioural inhibition at 24 months, and relevant physiological measures (heart rate and sinus arrhythmia) at 4 years of age with the aim to investigate to which extent both individual child temperament and parent–child relationship quality independently and/or interactively predicted physiological 'outcomes'. Interestingly, whereas no main effects of behavioural inhibition on the cardiac measures were found, children who displayed an avoidant attachment relationship had significantly higher heart period (lower heart rate) and higher respiratory sinus arrhythmia (RSA) than secure and anxious-resistant children at 4 years of age. However infants' avoidant attachment was not concurrently but only predictively associated with lower heart rate and high RSA at age 4 years. Therefore, an avoidant mother–child relationship in infancy could influence the development of an underaroused autonomic profile in early childhood. Recently, Schieche and Spangler (2005) investigated the influence of the quality of infant attachment and behavioural inhibition on adrenocortical responses in a sample of 76 toddlers during a primary non-attachment-related challenging context. They found that children who were not inhibited showed decreasing cortisol levels during the task situation, whereas highly inhibited children showed different patterns dependent on the quality of their attachment relationship. Specifically, among inhibited children those who were securely attached did not show adrenocortical activation whereas no reduction of cortisol levels were observed in insecure-ambivalent and disorganized toddlers. However, the findings of a recent investigation (Van Bakel and Riksen-Walraven, 2004) did not support the role played by attachment security as a moderating factor between unfavourable behavioural traits (social fearfulness) and cortisol reactivity. Similarly, Oosterman and Schuengel (2007) found no activation of the sympathetic nervous system in children who were insecurely attached and inhibited with regard to strangers.

In conclusion, most of the studies, with the exception of those just cited (Van Bakel and Riksen-Walraven, 2004, Oosterman and Schuengel, 2007), support the function of a secure attachment relationship as a social buffer against inappropriate behavioural dispositions capable to activate physiological responses to different stressful circumstances (such as the separation from the mother during the Strange Situation but also other emotionally challenging situations and tasks). In other words, security of attachment is likely to provide to the child the resources to reduce physiological activation even when the child's temperament may bias him or her to experience novel events as "potentially" threatening.

## Summary and conclusions

This chapter reviewed the impact of some environmental and biological factors on physiological reactivity to stress in childhood. The simplicity and non-invasiveness of measuring different stress indexes by new saliva assay techniques have greatly contributed to increment the number of psychobiological studies in child development research in recent years. The HPA axis functioning and salivary cortisol as the final product of this system have been widely investigated by many researchers. Recently, however, attention has turned to salivary alpha amylase as a new marker of the activity of SNS, which has led to a series of interesting studies that support its significant role in indexing the stress response both in adults and children. Environmental and biological factors which help to explain individual differences in HPA axis and SNS functioning in children have been examined, as they may serve as markers of risk for maladaptive developmental outcomes. Among environmental factors, a key role in buffering stress response may be played by the security of infant-mother attachment relationship, while child maltreatment appear to seriously affect the stress-related systems. However, the importance of some genetic polymorphisms and biologically-based temperamental disposition must also be considered in order to better understand the different outcomes in child's response to stress.

The current thesis presents the results of a study aimed at investigating the independent and interacting influences of genes and attachment relationships to psychophysiological responding in 1-year old infants. In the following chapters, after investigating the role played by some critical individual and methodological factors that could affect physiological stress reactivity measurements (chapter 2), the impact of environmental factors, biological factors and their relationships on HPA axis and SNS functioning will be tested. Specifically, the role played by individual differences in attachment pattern on stress response will be examined in chapter 3. The stress response will then be examined as a function of genes and temperament in chapter 4, while the interplay among genetic factors, temperament and attachment in relation to stress response will be investigated in chapter 5. The thesis will conclude with a discussion of the salient findings which may contribute to our understanding of the mechanisms involved in stress reactivity in childhood (chapter 6).

# CHAPTER 2

# POTENTIAL INTERFERING FACTORS IN MEASUREMENT OF THE STRESS RESPONSE

## Introduction

The growing interest in investigating activity of the stress-response systems during early development (see Gunnar, 2003) has been greatly facilitated by the simplicity and non invasivity measurement of some significant stress response parameters such as cortisol and alpha amylase through saliva. However, researchers who are interested in the examination of physiological stress indexes have to pay attention to several factors which might interfere with the reliability of the assessment if not adequately taken into account. In the following paragraphs, factors potentially affecting salivary cortisol and alpha amylase data, as significant indicators of the hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenergic-adrenomedulla (SAM) system functioning respectively, will be reviewed.

### *Factors which can interfere with the reliability of salivary cortisol and alpha amylase assessment*

The attention of researchers interested in the psychophysiology of stress has largely focused on salivary cortisol, whose investigation has incremented our understanding of the interactions among the HPA axis, environmental events, and behaviour in child development. Given the importance of this psychobiological parameter, a series of studies have looked into various factors related to individual, situational and methodological issues which might affect cortisol assessment. In contrast, the investigation of salivary alpha amylase as a significant measure of the SAM system has just recently captured the attention of researchers. Consequently, in-depth study of this new biomarker is in the beginning stages and the identification of those factors which might impact on the reliability of its assessment can only be speculated upon in most cases.

In the following sections, the sources of variations in basal cortisol and alpha amylase levels as well as those technical factors potentially interfering with their measurements will be examined, giving larger space to cortisol studies which have built up a stronger empirical evidence.

*Sources of variations in basal cortisol and alpha amylase levels*

Most of the existing studies examine one or more measures of salivary cortisol and alpha amylase before the onset of a stressor; however, in spite of the importance of basal cortisol and alpha amylase levels as points of reference, the picture of the development of these stress parameters in infants is still far from complete for cortisol and is hardly sketched for alpha amylase.

There are many factors affecting variations in basal cortisol in both adults and infants. As far as infants are concerned, the major sources of variation are linked to the circadian rhythm, age, and situational factors.

*Variation over the day and across age*

An important characteristic of the cortisol hormone is that it displays a circadian rhythm. While it is well documented in adults (Van Cauter and Turek, 1995), infants are born without a circadian rhythm and they acquire it during their first months of life. Studies differ in the exact age of appearance of the circadian rhythm with respect to cortisol: from as early as 2 months till the age of 9 months (Antonini et al, 2000; Kiess et al., 1995; Lewis and Ramsay, 1995; Mantagos et al., 1998; Onishi et al., 1983; Price et al., 1983; Santiago et al., 1996; Spangler, 1991). Also, even when the circadian rhythm has been found to be present, the nature of the rhythm emerged to be different than that of adults (de Weert and van Geert, 2002; Gunnar and Donzella, 2002). Gunnar and Donzella (2002), for example, conclude that significantly lower mid-afternoon than mid-morning levels (which are characteristic of adult curves) cannot be obtained reliably until children are around 4 years of age. In line with these authors, de Weert and van Geert (2002) did not find an important decrease in cortisol levels from morning to afternoon, as that exhibited in adults, in a longitudinal sample of 20 infants aged 5 to 8 months. Recently, Watamura and colleagues (2004) found that home baseline production of salivary cortisol decreased between 12 and 36 months, with children 30 to 36 months exhibiting lower levels of cortisol, particularly near wake-up, than children 12, 18, or 24 months of age.

Another interesting question is whether changes over age in basal cortisol levels and in reactions to stressors can be found in infants. Some studies have found a dampening in cortisol response to stressors from around 3–4 months of age till at least 15 months of age (Gunnar et al., 1996; Larson et al., 1998; Lewis and Ramsay, 1995). At the same time, no changes in basal cortisol were found between 7 and 15 weeks of age (Larson et al., 1998) and between 2 and 6 months of age (Gunnar et al., 1996), while a decrease in basal cortisol was found between two assessments at 6 and 15 months of age (Gunnar et al., 1996). In contrast, a decline in cortisol levels between 2 and 6 months of age and between the ages of 5 and 8 months was found by Lewis and Ramsay (1995) and de Weert and van Geert (2002) respectively. Recently, Watamura and colleagues (2004) observed significantly greater variability in cortisol levels at wake-up and midmorning among the 12-, 18-, and 24-month-olds than among the 30- and 36-month-olds children. However, across all of these ages a clear daytime rhythm in cortisol production emerge with the highest baseline values obtained near wake-up and the lowest values at bedtime.

As far as the investigation of salivary alpha amylase is concerned, not much is known about the circadian oscillations of this enzyme. In a recent study Nater and colleagues (2007) examined salivary alpha amylase diurnal profile in adults with hourly intervalled samplings from morning to evening in a naturalistic setting. They found a marked diurnal profile of salivary alpha-amylase activity with a pronounced decrease in the first 30 min after awakening, and steadily rising levels towards the afternoon and evening. Moreover, they did not find differences in the diurnal profile according to gender and physiological factors (such as body mass, activity level, cigarette smoking, food and drinks), whereas factors as age, stress and mood had an impact on the diurnal trajectory. Specifically, the increase of alpha amylase levels over time was less pronounced with increasing age, higher daily alpha amylase levels were associated with more chronic stress and stress reactivity, while momentary alpha amylase levels were higher when subjects reported higher calmness and positive mood in daily diaries.

Although no study has investigated alpha amylase diurnal profile in childhood and infancy, individual differences in basal alpha amylase levels were found to be associated with age of infants. In fact, salivary alpha amylase levels are very low in the first four months of life, have a sharp rise in the 0.9-1.9 year period and reach maximum levels by 5-6 years of age (O'Donnel and Miller, 1980). Recently, individual differences in alpha amylase activity were found also with age later in development (El Sheikh et al, 2005; Stroud et al., 2005, Susman et al., 2006).

*Situational factors: sleep, feeding, medication, locations and trips*

In general, basal cortisol levels are affected by various situational factors, such as daily hassles, physical condition and social factors (Ehlert et al., 1990; Flinn and England, 1995; Hellhammer and Wade, 1993; Kirschbaum et al., 1992). Thus, when assessing an individual's basal cortisol, the value obtained will be related to various external and internal factors. Accordingly, it will vary daily with the individual's situation. In adults, considerable day-to-day intra-individual variability in cortisol concentrations has been found (Kirschbaum and Hellhammer, 1989; Kirschbaum et al., 1990). Adult basal cortisol tends to be more stable and have more predictive validity with regard to socioeconomic and personality variables, and age, when it is taken in the morning hours (Brandtstädter et al, 1991).

In infants, studies have focused on the effects of some situational factors, namely sleep, feeding, medication, locations and trips on stress response. The main findings of these studies will be briefly reviewed.

**Sleep.** Relatively little is known about the neurobiology of daytime napping as it relates to regulation of the HPA system; however, some studies (Larson et al., 1991; Watamura et al., 2002) have suggested that the process of waking up from a nap stimulates the HPA system. This nap awakening stimulation may obscure the diurnal decrease in cortisol over the midportion of the day. In fact, there is some evidence that the mature pattern of a decrease in cortisol levels between midmorning and midafternoon might emerge as children give up their daytime naps (Watamura et al., 2004). In infants, daytime patterns of cortisol production fluctuate with daytime naps. Specifically, cortisol levels decrease while the infant naps and then rebound after the infant wakes up, returning to levels 45 min after the nap that are equivalent to prenap levels (Larson et al., 1991). Although Spangler (1991) found no relation between mean cortisol levels and the mean frequency of sleep and the mean duration of sleep per day and per episode, he did find that cortisol values were higher when a high amount of sleep had occurred in the hours preceding the assessment. Lewis and Thomas (1990) found similar associations: a negative correlation between cortisol level and the time awake for 6-month-old infants (but not for 2- and 4-month-olds).

On the other hand, Larson et al. (1991) found morning naps to be associated with decreases in cortisol in 9-month-old infants with returns to pre-nap levels 45 min after waking up. Last, de Weerth and van Geert (2002) found a negative effect of having slept during the 0.5 hr before the assessment thus suggesting the correction for sleep when analyzing infant cortisol data.

No studies have investigated the relationship between alpha amylase levels and napping.

**Feeding.** High cortisol levels have been found to be associated with a higher feeding latency (Gunnar et al., 1985). This is supported by Spangler (1991), who found first that a higher frequency of feeding episodes was associated with somewhat lower mean cortisol values, and second that cortisol levels decrease immediately after a feed and increase later on. Feeding behaviour thus seems to inhibit adrenocortical activity (Levine et al., 1989). However, there are studies that conclude that there appear to be no differences in pre- and post-feeding salivary cortisol levels (Lobo, 1990), while others state that the time elapsed since the last solid feeding (but not since the last liquid feeding) seems to correlate with basal levels of cortisol (Hertsgaard et al., 1992). This last finding about solid feeding is probably due to the post-prandial surge in cortisol which occurs approximately 45 min after the noonday meal (Riad–Fahmy et al., 1983).

Although no studies have investigated the relation between salivary alpha amylase levels and feeding, theoretically it could be present because of the role of salivary α–amylase in the digestion of carbohydrates and starches (Granger et al., in press). Specifically, increased salivary α–amylase activity might be found in saliva samples collected after consumption of high carbohydrate/starch meal.

**Medication.** Researchers who investigate HPA axis activity should pay attention to all those medications with the potential to act directly on the secretion or clearance of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol (e.g., compounds containing human, ovine, or synthetic glucocorticoids). Medications that alter subjective psychological experiences (e.g., antidepressants, narcotics) may indirectly influence salivary cortisol by changing the impact of environmental events on HPA axis activity. Other medications may indirectly influence salivary cortisol by affecting movement of cortisol from the circulation into the oral mucosa (e.g., blood thinners, vasoconstrictors), hydration, and the ability to generate sufficient saliva volume for assay (e.g., diuretics), or leaving residue in the oral cavity with potential to interfere with assay function (e.g., teething gels). Finally, medications with no mechanisms known to impact the HPA axis but that are very widely used require close examination just in case (e.g., teething gels, acetaminophen, acetylsalicylic acid). Surprisingly, there is just one study (Hibel et al., 2006) which has investigated the impact of medication status on cortisol levels in infants and adults. The purpose of this study was to describe associations between the use of common over-the-counter (OTC) and prescription medications with individual differences in salivary cortisol in a large sample of infants and their mothers. Saliva samples were collected before, 20 and 40 min after infants participated in a series of challenging tasks. A large percentage of infants (44%) had used at least one medication in the previous 48 h and most frequent were acetaminophen (e.g., Tylenol®) and cold medications (e.g., decongestants). Seven out of the 8 medication categories did not affect cortisol assessment, whereas acetaminophen did. Specifically, compared to infants not taking any medications, cortisol reactivity to the challenge tasks was less pronounced for infants taking acetaminophen. According to the authors this finding might have two possible explanations. The first one is related to the "side effects" of these medications which are used by parents to control and as a preventive measure against negative behaviours of infants (such as whining, fussing, cranking) (Allotey et al., 2004). The other possibility is linked to the fact that these medications are used to alleviate fever and/or inflammation which cause a significant increase of ACTH and cortisol levels.

The use of any prescription or OTC medication with potential to influence the para- or sympathetic nervous system (adrenergic agonists or antagonists) should be avoided in studies which involve the investigation of salivary alpha amylase. These medications include those used to control high blood pressure and angina. Similarly, OTC use of supplements with AAI properties should be cause for exclusion. Nicotine use (e.g., gum, patch, water) and tobacco smoke exposure should be avoided or controlled and the use of caffeine should be monitored (Granger et al., in press).

**Location and Trip.** Some studies reported different baseline salivary cortisol levels in infants across different sampling locations (Goldberg et al., 2003; Gunnar et al., 1989; Larson et al., 1991). Specifically, cortisol concentrations on arrival at the laboratory were found to be lower than those assessed at the same time of day at home in infants. These data seem to suggest that in laboratory studies with young children researchers examine cortisol responses to stressors starting from a level suppressed below typical baseline. Thus, some researchers recommend home sampling as a control for diurnal variation as well as employing a period of adaptation to the laboratory setting before introducing the stress experience (Gunnar and Talge, 2004). However, Goldberg and colleagues (2003) found significant correlations between home and laboratory cortisol concentrations although the means of the former were substantially higher than the latter.

Interestingly, Larson and colleagues (1991) demonstrated that the lower laboratory baselines are likely attributable to the car ride involved in transporting the infants to the laboratory, possibly because the ride has calming effects. In fact, they found that riding for 40 min in the car significantly lowered salivary cortisol concentrations and that this effect was obtained both for infants who did and who did not sleep during the car trip. No investigations have been carried out on the impact of sleeping and car ride on alpha amylase assay.

*Technical factors: the use of stimulants*

While highly reliable methods for saliva collection have been developed for older children and adults (see Kirschbaum and Hellhammer, 1989, 1994), such as chewing on a cotton plug or dental roll that absorbs saliva or chewing on a piece of gum and then expectorating saliva into a cup or vial, these collection procedures are not feasible with infants, toddlers, and preschoolers because they serve as choking hazards for young children. Absorbent cotton rolls that are too long to swallow can be used to avoid this choking hazard, but because the taste is unpleasant, many young children refuse to mouth them. Thus, many researchers have found that it is helpful to use a few grains of sweetened, drink-mix crystals containing citric acid (e.g., KoolaidTM) which are usually placed on the cotton roll in order to encourage young children to participate as well as to increase their saliva flow. However, evidence that KoolaidTM interferes with at least some cortisol assays (Schwartz et al., 1998) has led some researchers to strongly advise against their use. This, in turn, has led to a search for other candy-like saliva-stimulating substances that might increase child compliance without producing assay interference effects. SweetTartTM candies which contain malic, rather than citric acid do not interfere with an assay known to be highly sensitive to interference by KoolaidTM (Smider et al., 2002). In a recent study Talge and colleagues (2005) tested the effects of different quantities of SweetTartsTM and KoolaidTM in two commonly used assays. They found that, in general, oral stimulants did not affect the rank ordering of cortisol values but, depending on which assay was used, they increased or decreased the cortisol levels reported. Thus, oral stimulants should not be used with only a portion of the subjects in a study, nor should researchers assay stimulant-treated samples from the same study using different assays. When used sparingly, oral stimulants can be employed without compromising the quality of salivary cortisol data. No studies have investigated if the use of stimulants can affect the alpha amylase assay.

In this study, salivary cortisol and alpha amylase were sampled before the stressful procedure (Strange Situation; Ainsworth et al., 1978) as well as at 20 and 40 minutes following the stressor with the aim to investigate if some individual, situational and technical factors may affect these physiological measurements. Specifically, child and family demographic characteristics (i.e. gender, age, socio-economical level etc), child's state variables (i.e. sleeping, feeding, medication etc) and technical factors such as the use of stimulants, which have been found to affect or may theoretically affect salivary cortisol and alpha amylase levels, were examined as potential interfering variables with HPA and SAM activity.

## Methods

*Participants*

The initial sample was composed of 82 healthy infants (45 boys and 37 girls) aged 12 to 18 months (mean age = 14.6; SD = 1.8) and their mothers who gave their written consent for participating in the study. 68.3% of the sample was recruited through poster advertisements in day care, swimming courses and other services for young families and the remaining 31.7% through a list of families who had previously taken part in another research project at the "Eugenio Medea" Scientific Institute and had given their availability to participate in other studies on childhood development. Most of parents were Italian, married and belonged to middle-high social class levels; their mean age was 34.2 years (SD = 4.3) for mothers and 36.8 years (SD = 5.1) for fathers. Infants did not have any significant pathologies at birth and none suffered of asthma at the time of assessment. Around 70% of infants were first born; 2 of them (2.4%) were preterms but their weight at birth was higher than 2 kilos thus highly minimizing the risks associated with prematurity. 8 (9,7%) and 6 (7,3%) infants were not included in the present analyses because they did not have any cortisol and alpha amylase data respectively. The lack of data was caused by insufficient saliva production by the child or by the child's refusal to cooperate with the saliva sampling. 23 (28%) and 21 (25,6%) had incomplete cortisol and alpha amylase data respectively because: a) for 10 infants out of them saliva was not collected 40 min post stressful procedure as the decision to extend the saliva collection to 40 min post stressor was taken after the beginning of the study in the light of newly published research findings (Goldberg et al., 2003), and b) for the remaining infants the volume of saliva was insufficient for the assays.

The final sample included 74 and 76 infants with cortisol and alpha amylase data respectively. 51 out of 74 and 55 out of 76 had all cortisol and alpha amylase samples whereas 50 infants had both completed cortisol and alpha amylase data. Infants of the three groups (all – partial – missing cortisol and alpha amylase data) did not differ on any of the study variables with the exception of the following ones: "time of wake up" for cortisol data, and "hours of sleep last night" and "disruptions in sleep" for alpha amylase data. Specifically, infants who were not willing or refused to cooperate in saliva collection woke up later ($\chi^2$ (2) = 5,91; $p$ = 0.05) in comparison with infants who cooperated. Further, infants who were not willing or refused to cooperate in saliva collection were more likely to have disruptions in sleep during the night before the assessment ($\chi^2$ (2) = 6.52; $p$ = 0.04) in comparison with infants who cooperated; coherently, infants who did not cooperate had the lowest mean of hours slept the night before the assessment ($F$ = 3.38; $p$ = 0.04). To test these differences among participants, further analyses on infants with all physiological data versus infants with incomplete physiological data were performed. Again, infants who had incomplete cortisol data woke up later than infants with all cortisol data ($\chi^2$ (1) = 5,74; $p$ = 0.02) as well as infants who had incomplete alpha amylase data were more likely to have "disruptions in sleep" before the assessment than infants with all alpha amylase data ($\chi^2$ (1) = 5.91; $p$ = 0.01). No significant differences related to the mean of the hours slept the night before the assessment were found between infants with complete and partial alpha amylase data.

Table 2.1 summarizes the demographic characteristics of the final samples.

*Procedure*

The study protocol was approved by the Ethic Committees of University College London and the Scientific Institute "Eugenio Medea". The assessment procedure took place in the Psychopathology Unit of the Scientific Institute "Eugenio Medea", where the assay of salivary cortisol was also performed. To control for diurnal variation in basal cortisol concentrations, all assessments were scheduled in the morning and, whenever it was possible, at the same time of day (approximately between 9.00 and 11.00 a.m). On arrival at the laboratory, mothers read and signed the consent forms and the first (baseline) saliva collection was performed as soon as possible.

Mothers also completed a brief socio-demographic form and provided information regarding those factors which can affect basal cortisol and alpha amylase levels. The priority during the laboratory session was to be minimally disruptive of the infant's normal periods of alertness, naps, and meal schedule. Thus, we proceeded to the stressful procedure (Strange Situation, SS) as quickly as possible. Saliva was sampled immediately before the stressor as well as 20 and 40 min after it. These time points allow adequate time for the response to the SS to be reflected in salivary cortisol concentrations (Goldberg et al., 2003). The exact time of each collection was recorded. After the stressor and between saliva collections, mother–infant pairs could remain in the laboratory playroom or go outside if they preferred.

Table 2.1

*Socio-demographic characteristics of the two samples*

| | CORTISOL DATA *f* | CORTISOL DATA *%* | A-A DATA *f* | A-A DATA *%* |
|---|---|---|---|---|
| **CHILD'S DEMOGRAPHIC CHARACTERISTICS** | | | | |
| **GENDER** | | | | |
| *Boys* | 40 | 54,1 | 41 | 53,9 |
| *Girls* | 34 | 45,9 | 35 | 46,1 |
| **AGE** | | | | |
| *12-14 months* | 36 | 48,6 | 36 | 47,4 |
| *15-18 months* | 38 | 51,4 | 40 | 52,6 |
| **BIRTH ORDER** | | | | |
| *First born* | 50 | 67,6 | 52 | 68,4 |
| *Second born or third born* | 24 | 32,4 | 24 | 31,6 |
| **FAMILY DEMOGRAPHIC CHARACTERISTICS** | | | | |
| **MATERNAL AGE** | | | | |
| *< 35 years* | 47 | 61,8 | 49 | 64,5 |
| *> 35 years* | 27 | 38,2 | 27 | 35,5 |
| **PATERNAL AGE** | | | | |
| *< 35 years* | 27 | 38,2 | 29 | 38,2 |
| *> 35 years* | 47 | 61,8 | 47 | 61,8 |
| **NATIONALITY** | | | | |
| *Italian* | 67 | 90,5 | 69 | 90,8 |
| *Both parents not Italian* | 4 | 5,4 | 4 | 5,3 |
| *Mother or father not Italian* | 3 | 4,1 | 3 | 3,9 |
| **MATERNAL EDUCATION *** | | | | |
| *Less than 10 years* | 12 | 16,2 | 12 | 16 |
| *More than 10 years* | 61 | 82,4 | 63 | 84 |
| **PATERNAL EDUCATION *** | | | | |
| *Less than 10 years* | 17 | 23,3 | 17 | 22,7 |
| *More than 10 years* | 56 | 76,7 | 58 | 77,3 |
| **SES *** | | | | |
| *Low* | 6 | 8,3 | 6 | 8,1 |
| *Medium* | 31 | 43,1 | 32 | 43,2 |
| *High* | 35 | 48,6 | 36 | 48,6 |
| **INCOME *** | | | | |
| *From 10,000 – 31,000 Euros* | 27 | 40,9 | 27 | 39,7 |
| *More than 31,000 Euros* | 39 | 59,1 | 41 | 60,3 |
| **PARENTAL STATUS** | | | | |
| *Married* | 66 | 89,2 | 68 | 89,5 |
| *Co-habitants* | 8 | 10,8 | 8 | 10,5 |
| | *mean* | *SD* | *mean* | *SD* |
| **CHILD'S AGE** | 14,53 | 1,83 | 14,56 | 1,83 |
| **MATERNAL AGE** | 34,35 | 4,48 | 34,32 | 4,43 |
| **PATERNAL AGE** | 37,15 | 5,27 | 37,07 | 5,23 |
| **SES** | 63,26 | 19,52 | 63,17 | 19,33 |

* some missing data

*Instruments and psychophysiology assessment*

**Socio-demographic form.** In order to collect socio-demographic variables, an ad hoc form to be filled out by parents was designed. This form included demographic data (child's gender and age, birth order, number of brothers/sisters, parents' marital status, place of residence), variables related to the family socio-economic status (mother's and father's educational level and employment, annual income). Parents had also to specify if they were biological or adoptive parents. Only for families coming from foreign countries, the nationality and the number of the years of residence in Italy was collected. Socio-economic status (SES) was evaluated using two indicators: the parents' employment and the family annual income. In the first case, it was coded according to information provided by parents, on the basis of the Hollingshead (1975) 9-point scale for parental occupation. A score (from 1 to 9) was assigned to each job; the highest of two scores was used when both parents were employed. Our SES scoring system is based on the Hollingshead scores converted to two digits (i.e. Hollinghshead 1-9 becomes 10-90), so that for unclear occupations a mean value of the two most likely levels was used. Scores ranging from 70-90 corresponded to the upper status, scores ranging from 40-65 correspond to the middle status, scores ranging from 10-35 corresponded to the lower status, while a score of 0 is used when information was provided but cannot be scored (i.e. housewives; self-employed people with no other information; retired). In the second case, the income levels were defined on the basis of using the criteria defined by the Italian law for tax declaration (IRPEF, Gazzetta Ufficiale, 2001).

**Stressor.** The Strange Situation procedure (Ainsworth et al. 1978) was used as stressor. It is a structured observational procedure consisting of seven 3-minute episodes in which the infant is alone, with mother, with a female stranger, or with both mother and stranger, in an unfamiliar setting. These episodes are ordered to increase stress in standardized increments that are manageable for the baby. There are two separations from the mother, one in which the infant is with the stranger and one in which the infant is first alone and then with the stranger before the mother returns. Separations are curtailed if the baby cries hard for more than 30 seconds (for a brief description of each episode see table 1.2 in Chapter 1).

The Strange Situation procedure is a widely employed procedure in infant studies because it allows to investigate children's reactions to separation from caregiver which represents a natural stressor in infancy. Behavioural and physiological responses to the SS have been examined by psychobiologists interested in the investigation of infants' reaction to stress (i.e. Nachmias et al, 1996; Schieche and Spangler, 2005; Spangler and Grossman, 1993).

**Form on factors which are related to basal cortisol and alpha amylase.** In order to collect information on factors related to child's sleep, food, physical conditions and daily experiences which can interfere with basal cortisol and alpha amylase assessment, an ad hoc form to be filled out by parents was designed. This form includes questions concerning: 1) the frequency and the duration of sleep (time of wake up; number of hours slept the night before; disrupted sleep); 2) when the child has had the last meal; 3) his/her actual physical condition, if the child suffers of asthma and if he/she takes some medications (if yes, which ones); 4) the duration of trip to arrive to the laboratory and his/her mood during the trip; 5) every events causing anxiety and distress happened before the salivary cortisol collection. The time of saliva collection before and after the SS was also recorded.

Table 2.2 shows some descriptive statistical analyses of child's state variables potentially interfering with cortisol and A-A data in both samples.

Table 2.2

*Child's state variables of the two samples*

| | *Cortisol data* | | *A-A data* | |
|---|---|---|---|---|
| | *F* | *%* | *f* | *%* |
| **CHILD'S STATE VARIABLES** | | | | |
| **Time of saliva collection** | | | | |
| *h. 9:00 – 11: 00* | 54 | 73 | 55 | 72,4 |
| *h. 11:00–13: 00* | 20 | 27 | 21 | 27,6 |
| **Time to fall asleep the night before** | | | | |
| *0-15 minutes* | 55 | 74,3 | 55 | 72,4 |
| *More than 15 minutes* | 19 | 25,7 | 21 | 27,6 |
| **Disruptions in sleep** | | | | |
| *Not Disrupted sleep* | 23 | 31,1 | 23 | 30,3 |
| *Disrupted sleep* | 51 | 68,9 | 53 | 69,7 |
| **Medication** | | | | |
| *Not medication* | 63 | 85,1 | 65 | 85,5 |
| *medication* | 11 | 14,9 | 11 | 14,5 |
| **Sleep during the trip** | | | | |
| *No sleep* | 60 | 81,1 | 62 | 81,6 |
| *sleep* | 14 | 18,9 | 14 | 18,4 |
| | *Mean* | *SD* | *Mean* | *SD* |
| **Hours of sleep last night** | 9,80 | 0,91 | 10,13 | 1,22 |
| **Time of wake up** | 7,40 | 0,74 | 7,40 | 0,75 |
| **Time from awakening to assessment** | 1,99 | 0,81 | 1,99 | 0,80 |
| **Time from breakfast to assessment** | 1,76 | 0,77 | 1,75 | 0,77 |
| **Duration of trip** | 26,39 | 17,17 | 26,16 | 17,00 |
| **Minutes of sleep during the trip** | 4,51 | 11,53 | 4,80 | 11,84 |

*Salivary cortisol and alpha amylase collection*

A sterile 6-in. cotton dental roll was used for saliva collection. The tip of the cotton roll was lightly dusted with Kool Aid crystals (cherry flavor), and was placed in the child's mouth. The amount of Kool-Aid crystals did not exceed 3 grains. The child was encouraged to chew on the roll until it was saturated with saliva and no visible crystals remained on the surface of the roll. If the child was reluctant to comply, the help of the mother was enlisted. Although concern has been expressed about the use of Kool-Aid due to the possibility of artificial elevation in cortisol levels (Schwartz et al., 1998), this does not appear to pose a problem here because we used very small quantities of Kool Aid. Saturated cotton rolls were placed into a needleless syringe and saliva was expressed into a cryogenic vial. When children refused to chew on the cotton roll, a different method of saliva collection, consisting of a microsponge to be placed in the infant's mouth, was used for limiting the loss of data. The microsponge, technically called a Sorbette (Salimetrics), was placed in the mouth and moved around in the mouth and lip area to collect saliva until it began to expand (at least one minute total time). The plastic shaft of the Sorbette was cut in half and inserted into a 2mL cryovial with the absorbent end up. After the end of the assessment procedure, all saliva samples were stored frozen at – 80° C until being assayed. To extract the saliva from sorbettes, the cryovials were centrifuged for 20 minutes at 1500 Xg before the assays. Salivary cortisol assay was performed at the biology laboratory of the Scientific Institute "Eugenio Medea" whereas for alpha amylase assay saliva samples were shipped in dry ice to a Pennsylvannia laboratory (Salimetrics), highly specialized for this kind of assay.

*Salivary Cortisol Assay*

The assessment of cortisol levels was initially done by the Salimetrics HS-Cortisol EIA Kit, a competitive immunoassay specifically designed for the quantitative measurement of salivary cortisol. A small number of saliva samples (N = 10) was re-assayed in the Pennsylvannia laboratory in order to verify the reliability of the kit and laboratory procedure. Correlation coefficients calculated on the same saliva samples assayed in the two labs were $r$ = 0.99 ($p$ <0.0001) and $r$ = 0.98 ($p$ <0.0001) for pre and post Strange Situation respectively, thus providing strong support for the reliability of the cortisol assay performed in the Italian lab. Subsequently, a new updating of the kit for cortisol assay (Expanded Range (ER) HS-Cortisol EIA Kit, Salimetrics) was employed as it replaced the previous one. The improvements include a new monoclonal antibody, lower cross-reactivity, improved reliability, as well as an expanded upper range of the calibration curve. The results of the two assays are nearly perfect linear duplicates of each other, $r$ (106) = 0.99, $p$ < 0.001 as tested by researchers of Salimetrics on 108 saliva samples. Cortisol values assayed with the previous kit (N = 34 infants) were transformed according to the following formula: Expanded Range HS-cortisol = ((-0.02293) + (HS cortisol *0.954)) with the aim to compare findings obtained with the two kits.

All samples from one infant were analyzed in one assay to minimize the variability of the results. To guarantee validity of analysis, duplicate assays were performed whenever possible. Some samples with a volume of less than 25 ml were too small for duplicate assay. In these cases, single assays were accomplished.

All samples were assayed for salivary cortisol using a highly sensitive enzyme immunoassay US FDA (510k) cleared for use as an in vitro diagnostic measure of adrenal function (Salimetrics, State College, PA). The test uses 25 ml of saliva (for singlet determinations), has a reported sensitivity of 0.003 µg/dL. Average intra-and interassay coefficients of variation were less than 9% and 10% respectively.

*Salivary Alpha Amylase Assay*

Samples were assayed for Alpha Amylase (A-A) using a commercially available kinetic reaction assay (Salimetrics, State College, PA). The assay employs a chromagenic substrate, 2-chloro-p-nitrophenol, linked to maltotriose. The enzymatic action of A-A on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405nm using a standard laboratory plate reader. The amount of A-A activity present in the sample is directly proportional to the increase (over a 2 min period) in absorbance at 405 nm. Results are computed in U/mL of A-A using the formula: [Absorbance difference per minute_total assay volume (328 ml)_dilution factor (200)]/[millimolar absorptivity of 2-chloro-p-nitrophenol (12.9) _sample volume (.008 ml)_light path (.97)]. The test has a reported average inter assay variation computed for the mean of average duplicates for 8 separate runs for lower (10,6 U/mL) and higher (166.0 U/mL) concentrations samples were 5,8 and 3,6 % respectively whereas intrassay variation computed for the mean of 10 replicate tests of low (17,7% U/mL), medium (108,8 U/mL), and high (474,6 U/mL) concentrations samples were 7,2%, 6,7%, and 2,5%, respectively (Granger et al, in press).

*Statistical analysis*

Data inspection revealed that cortisol and A-A concentrations were not normally distributed. Therefore their values were $\log_{10}$ and $\log_{10} + 1$ transformed for cortisol and A-A respectively. Of the original 74 and 76 participants with cortisol and A-A data respectively, one was excluded as outlier beyond 3 SD above the mean for all cortisol data.

The effects of potential interfering variables on stress reactivity were tested by hierarchical multivariate linear model (HMLM) analyses. The advantage of hierarchical linear modelling, in addition to its flexibility in handling a variety of different study designs and types of predictor, is that it allows for the modelling of repeated measures data where some cases have one or more missing observations (Raudenbush et al., 2000). HMLM analyses involve regression-type models of data at two levels: variations between repeated observations nested within persons (level 1) and variations between individuals (level 2). In the current study, the level-1 regression model simply modelled the repeated observations as a function of three terms representing the intercept (the initial level), linear slope (representing linear increases or decreases) and quadratic slope (representing curvature). These parameters then became the dependent variables that were modelled by predictors at level 2 (person-level variables, such as attachment, or other covariates). Thus, the significance of level 2 variables represents the extent to which the intercept or slopes vary reliably according to the level 2 predictors, and therefore are equivalent to between-subjects x within-subjects interaction terms in traditional ANOVA. The model also provides estimates of error variance at each level: random error at level 1 and systematic error at level 2 (i.e. consistencies in the repeated observations within an individual that are not explained by level 2 predictors). The model can be represented formally as follows: in the level -1 model the outcome for case *i* within unit *t* is modelled as:

$$Y_{it} = \beta_{oi} + \sum_{p=1}^{P} \beta_{pi} X_{pt} + r_{it}$$

where:

$\beta_{0i}$ *(0 = 0,1,...,O)* are level -1 intercepts

$\beta_{pi}$ *(p = 0,1,...,P)* are level -1 coefficients;

$X_{pt}$ the level-1 predictor p for case t;

$r_{it}$ *is the level-1 random effect; and*

$\sigma^2$ *is the variance of* $r_{it}$ that is the level-1 error variance

while level-2 model includes covariates, Xi, that vary between persons:

$$\beta_{pi} = \beta_{pO} + \sum_{q=1}^{q} \beta_{pq} X_{qi} + r_{qi}$$

The following variables nationality, parental status, child's health, mood during the trip, and any stressor prior to assessment were excluded from the analyses because of their small sample size.

## Results

*Preliminary analyses*

Table 2.3 shows means, SDs and ranges for raw and log-transformed values of salivary cortisol and A-A before as well as 20 and 40 minutes after the SS whereas figure 2.1 illustrates the overall response in cortisol and A-A levels. Univariate ANOVA for repeated measures revealed a significant increase of A-A concentrations from pre to post SS ($F = 6{,}76$; $p = 0.002$) whereas no significant differences emerged for cortisol values ($F = 0{,}42$; $p = 0{,}61$).

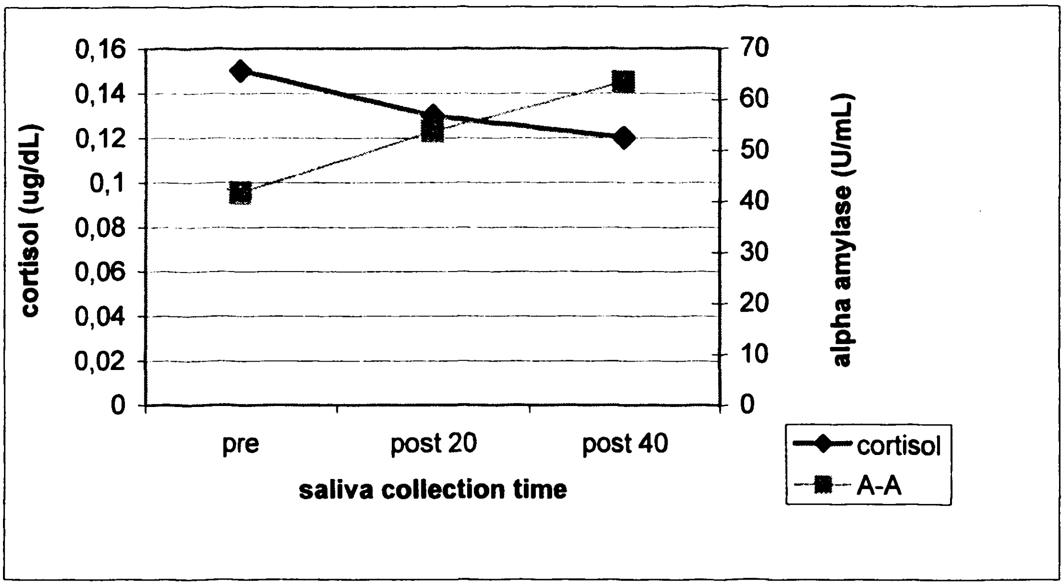
Table 2.3

*Means, SDs and ranges for raw and log-transformed cortisol and A-A concentrations*

| Measure | n | Raw Mean (SD) | Raw Range | Log-Transformed Mean (SD) | Log-transformed Range |
|---|---|---|---|---|---|
| T1 Pretest Cortisol in μg/dl | 68 | 0,15 (0,10) | 0,02-0,51 | -0,92 (0,29) | -1,82 -0,29 |
| T2 Post test Cortisol in μg/dl | 65 | 0,13 (0,09) | 0,01-0,45 | -0,97 (0,33) | -2,24 -0,35 |
| T3 Post test Cortisol in μg/dl | 58 | 0,12 (0,08) | 0,03-0,31 | -0,98 (0,26) | -1,47 -0,51 |
| T1 Pretest A-A in U/mL | 71 | 41,71 (31,31) | 1,10-115,9 | 2,47 (0,41) | 1,04-3,06 |
| T2 Post test A-Amylase in U/mL | 71 | 53,74 (49,24) | 1,00-231,1 | 2,56 (0,42) | 1,00-3,36 |
| T3 Post test A-A in U/mL | 60 | 63,35 (42,80) | 0,70-212,2 | 2,68 (0,38) | 0,85-3,33 |

Figure 2.1

*Salivary Cortisol and A-A levels (raw mean) from pre to post stressor*




Further, an individual differences approach was considered by which infants who had an increase of post stressor salivary cortisol and A-A levels equal or above 10% in respect with the baseline were considered to be physiologically reactive to the stressful procedure. Most of the infants had an increase of cortisol and A-A levels post stressor (see table 2.4) when 10% difference from pre SS was used as a criterion for change. For A-A data, more infants than expected showed an activation of the SAM system ($\chi^2$ (1) = 12.86; $p < 0.001$).

To analyze correlations between salivary cortisol and A-A levels, Spearman correlation coefficients were computed. Table 2.5 shows Spearman coefficients among cortisol and A-A values before as well as 20 and 40 min post SS. Interestingly, cortisol and A-A levels were not correlated for all the three saliva collection times.

Table 2.4

*Frequencies and percentages of infants who showed an increase of cortisol and A-A concentrations using 10% difference from pre-stressor as a criterion for change*

| | No increase post stressor | Increase 20 **and** 40 min post stressor | Increase 20 **or** 40 min post stressor |
|---|---|---|---|
| | Cortisol | | |
| F (%) | 28 (42.4) | 20 (30.3) | 18 (27.3) |
| | Alpha Amylase | | |
| F (%) | 20 (28.6) | 30 (42.9) | 20 (28.6) |

Table 2.5

*Correlations among cortisol and A-A values before and after the stressor*

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **1 AA – Pre SS** | - | | | | | |
| **2 AA – Post 20 min** | 0.67*** | - | | | | |
| **3 AA – Post 40 min** | 0.70*** | 0.58*** | - | | | |
| **4 cortisol – Pre SS** | -0.10 | -0.05 | 0.09 | - | | |
| **5 cortisol – Post 20 min** | -0.03 | -0.02 | 0.13 | 0.23 | - | |
| **6 cortisol – Post 40 min** | 0.05 | 0.12 | 0.01 | -0.06 | 0.82*** | - |

* $p<0.05$ ** $p < 0.01$ *** $p < 0.001$

*The impact of socio-demographic factors*

In order to test which socio-demographic factors were able to predict cortisol and A-A levels, separate HMLM analyses were conducted. As shown in table 2.6, no demographic variables predicted significantly salivary cortisol and A-A reactivity with the exception of birth order for cortisol values. Specifically, second and third born infants had an increase of cortisol levels after the SS in comparison with first born infants who had a decrease of cortisol levels after the stressor (see figure 2.2).

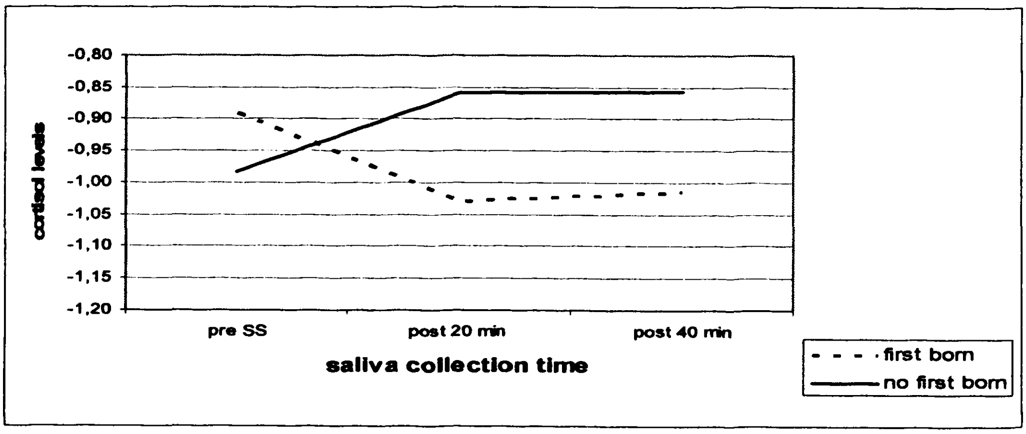
Table 2.6

*Socio-demographic factors as predictors of cortisol and A-A reactivity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| Gender | -1.05 | -1.05 | 1.35 |
| Age | 0.70 | 1.24 | -1.79 |
| Birth order | -1.27 | **2.70**** | **-2.12*** |
| Maternal age | -0.93 | 1.27 | -0.93 |
| Paternal age | -0.37 | 0.95 | -0.79 |
| Maternal education | -1.23 | -0.39 | 0.30 |
| Paternal education | -0.61 | 0.25 | 0.08 |
| SES | -1.23 | 0.31 | 0.06 |
| Income | 0.42 | 1.03 | -1.46 |
| | | Alpha Amylase | |
| Gender | 0.83 | 0.36 | -0.24 |
| Age | 1.12 | 0.67 | -1.08 |
| Birth order | -0.85 | -1.75 | 1.82 |
| Maternal age | 0.86 | -0.24 | 0.26 |
| Paternal age | -0.002 | -0.59 | 0.54 |
| Maternal education | -0.54 | -0.14 | -0.06 |
| Paternal education | 1.33 | -0.36 | 0.02 |
| SES | -0.001 | -0.40 | 0.40 |
| Income | -0.20 | -0.95 | 0.93 |

* $p<0.05$ ** $p < 0.01$ *** $p < 0.001$

Figure 2.2 *The effect of birth order on salivary cortisol log-transformed concentrations (μg/dl)*



*The impact of child's state factors*

In order to test which child's state factors were able to predict cortisol and A-A levels, separate HMLM analyses were conducted. As shown in table 2.7, the following factors were found to be predictive of cortisol levels: "time from awakening to assessment", "time from breakfast to assessment", "medication" and "duration of trip". In figure 2.3, the direction of each significant effect on stress response is shown in different panels. The strongest predictor of cortisol was "time from awakening to assessment". Specifically, infants who were assessed after a shorter time from awakening had higher cortisol baseline levels and showed a decrease of cortisol levels post SS in contrast to infants assessed after a longer time who showed a slight cortisol increase (Fig 2.3 panel A) from pre to post SS. In addition, infants who were assessed after a shorter time from breakfast had higher cortisol baseline levels and showed a decrease of cortisol levels 20 min after the SS and a slight increase 40 min post SS in contrast to infants assessed after a longer time from breakfast who showed the opposite trend (Fig 2.3 panel B). Infants who took medications had lower cortisol baseline levels than infants who did not take medications (Fig 2.3 panel C). Last, infants who had a shorter trip to the lab showed a higher decrease of cortisol levels after 20 min from the end of the SS followed by an increase at 40 min post SS in comparison with infants who had a longer trip (Fig 2.3 panel D).

Table 2.7

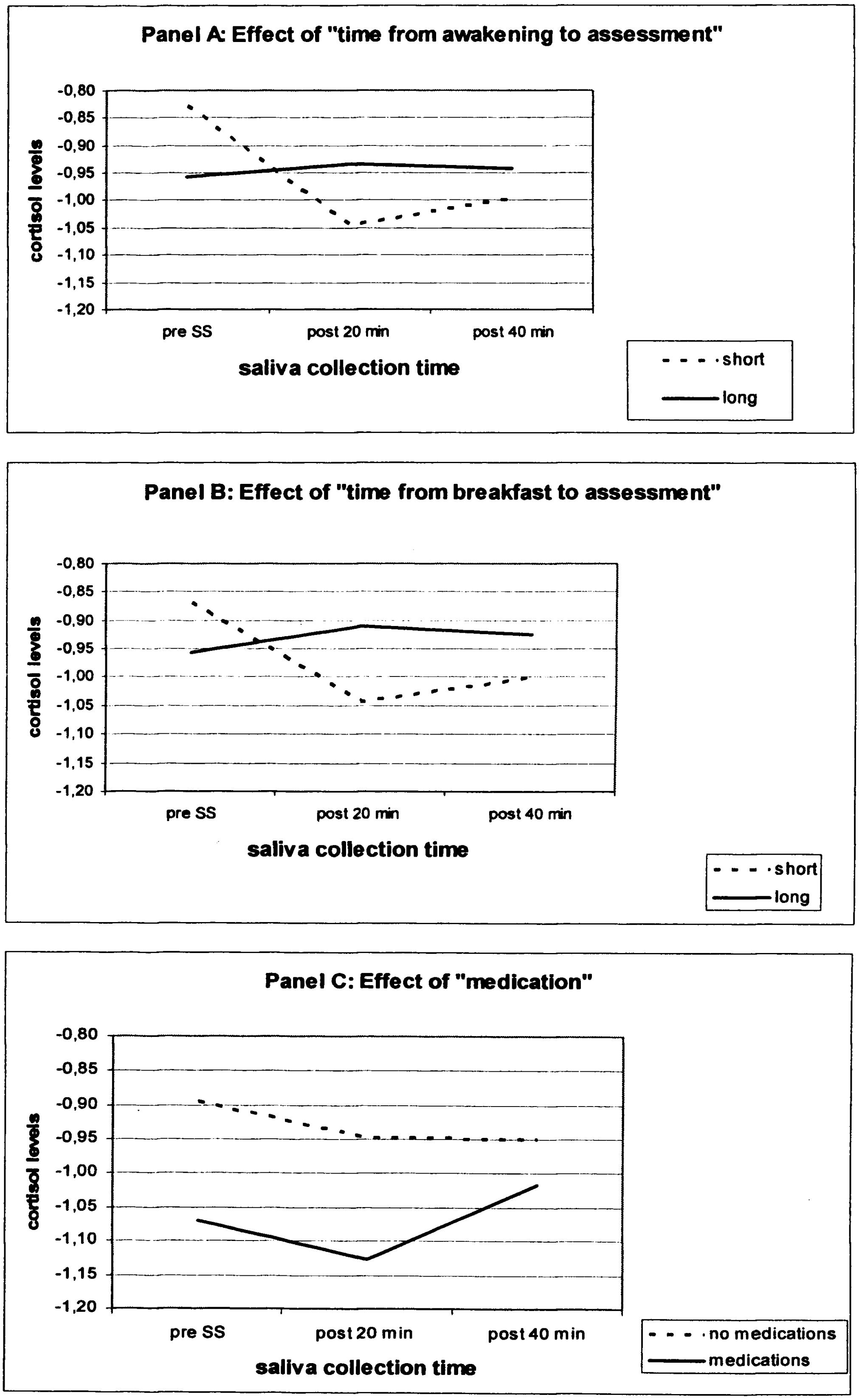
*Child's state factors as predictors of cortisol and A-A reactivity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| Time to fall asleep the night before | -1.30 | 0.63 | -0.47 |
| Disruptions in sleep | 0.29 | -0.38 | 0.42 |
| Hours of sleep last night | 1.57 | -0.50 | 0.14 |
| Time from awakening to assessment | **-3.08**** | **4.01***** | **-3.43**** |
| Time from breakfast to assessment | **-2.03*** | **3.48***** | **-3.05**** |
| Medication | **-2.03*** | -0.37 | 0.82 |
| Duration of trip | -0.04 | 1.78 | **-2.38*** |
| Sleep during the trip | 0.76 | 0.64 | -1.21 |
| Minutes of sleep during the trip | -1.31 | 1.01 | -1.15 |
| | | Alpha Amylase | |
| Time to fall asleep the night before | 0.35 | 1.13 | -1.21 |
| Disruptions in sleep | -0.98 | **2.46**** | **-2.58**** |
| Hours of sleep last night | 0.52 | 0.90 | -0.84 |
| Time from awakening to assessment | 1.02 | **-1.98*** | 1.67 |
| Time from breakfast to assessment | 0.72 | **-2.88**** | **2.66**** |
| Medication | 0.91 | **-2.53**** | **-2.34*** |
| Duration of trip | 0.15 | **-2.14*** | **2.19*** |
| Sleep during the trip | -1.05 | -0.27 | 0.60 |
| Minutes of sleep during the trip | 0.09 | -0.75 | 0.63 |

* $p<0.05$ ** $p < 0.01$ *** $p < 0.001$

Figure 2.3

*Significant effects of child's state factors on salivary cortisol log-transformed concentrations (µg/dl)*



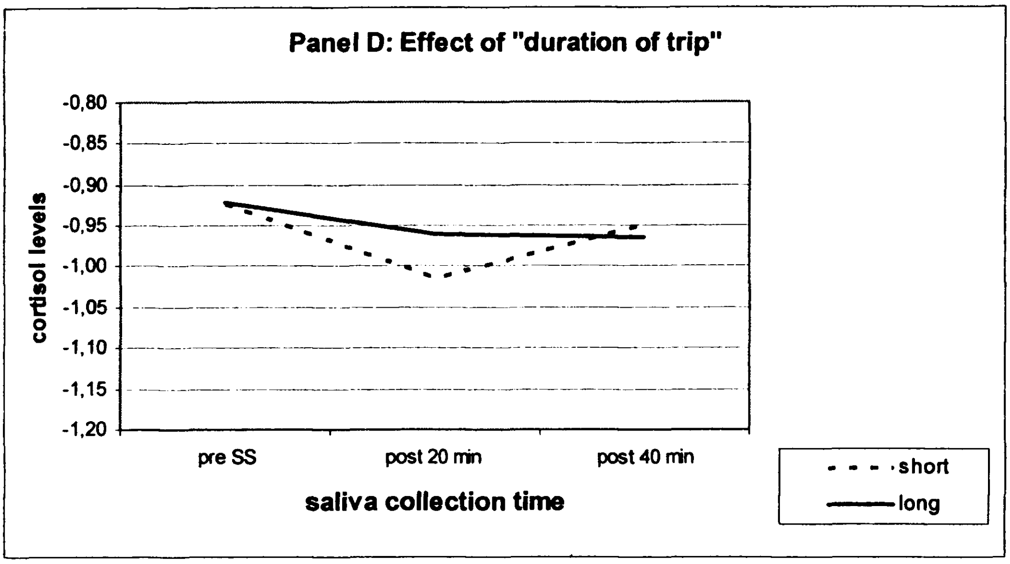
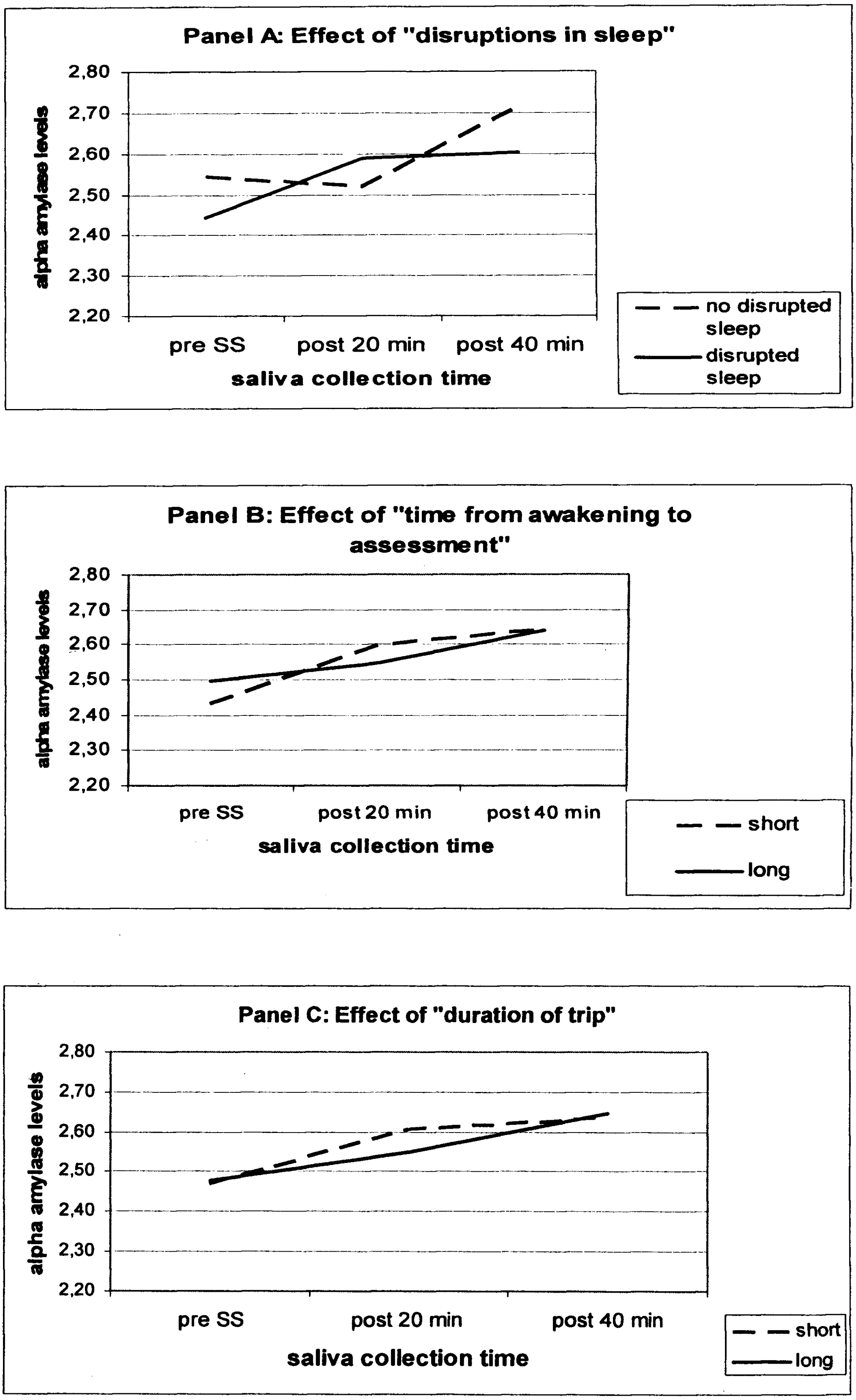
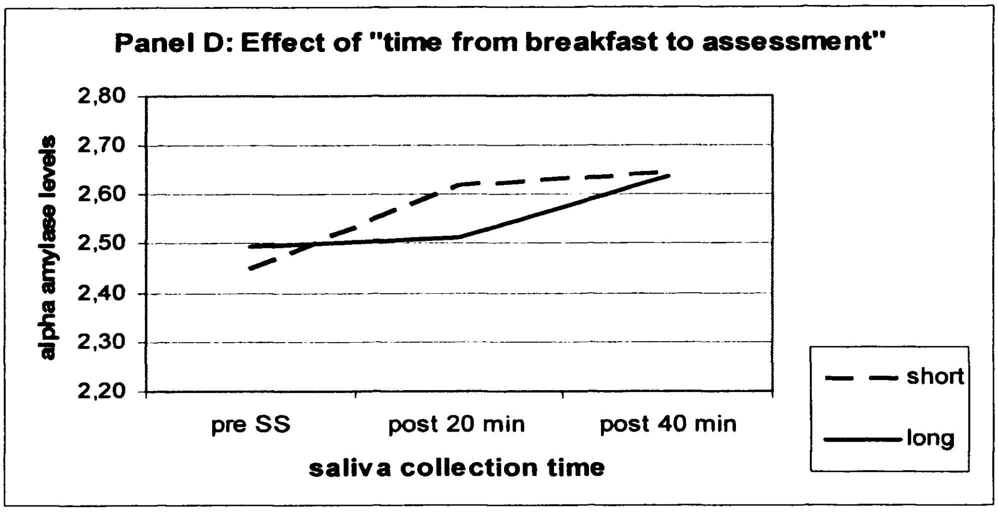


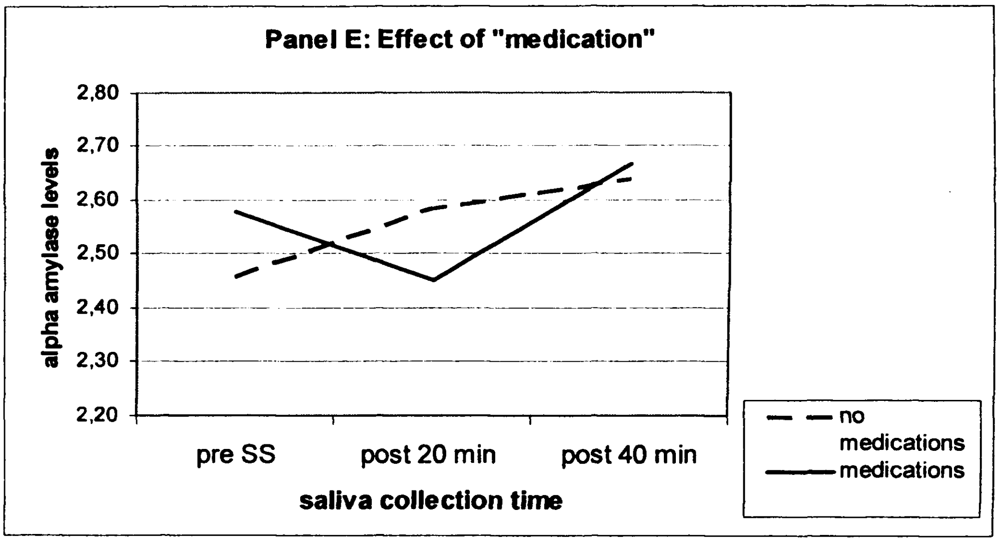

Table 2.7 also shows the results of HMLM analyses for A-A data, and the direction of the effects is displayed in figure 2.4. “Disruptions in sleep”, “time from awakening to assessment”, “duration of trip” , “time from breakfast to assessment” and “medication” variables significantly predicted A-A reactivity. Specifically, infants who had slept the whole night before the assessment showed an increase of A-A levels from pre to 40 min post the SS in comparison with infants who had disrupted sleep who showed an increase of A-A levels from pre to 20 min post the SS (Fig 2.4 panel A). Infants who were assessed after a shorter time from awakening had higher A-A levels post SS than infants assessed after a longer time (Fig 2.4 panel B). Similarly, infants who had a shorter car trip showed higher A-A reactivity than infants who had a longer trip (Fig 2.4 panel C). Further, infants who had had their breakfast in a shorter time from assessment showed higher A-A reactivity than infants who had had their breakfast in a longer time from assessment (Fig 2.4 panel D). Last, infants who took medications had a decrease of A-A levels after 20 minutes from the SS in contrast with infants who did not take medications who showed A-A reactivity both after 20 and 40 minutes (Fig 2.4 panel E).

Figure 2.4

*Significant effects of child's state factors on salivary A-A log-transformed concentrations (U/mL)*






In order to test which factors, among those found to be associated with cortisol reactivity, had significant independent effects on stress response, "time from awakening to assessment", "time from breakfast to assessment", "medication" and "duration of trip" were entered together into the HMLM model. As shown in table 2.8, cortisol levels were found to be independently predicted by "time from awakening to assessment" and "duration of trip".

Similarly, "disrupted sleep", "time from awakening to assessment", "time from breakfast to assessment", "medication" and "duration of trip" were entered together into the HMLM model in order to analyze which of them had significant independent effects on A-A levels. None of these factors was found to independently predict SAM system activation with the exception of the use of medications (table 2.8).

Table 2.8

*Predictors of cortisol and A-A response*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| Time from awakening to assessment | **-2.43**** | **1.95*** | -1.67 |
| Time from breakfast to assessment | 0.76 | 0.82 | -0.70 |
| Medication | -1.76 | -1.16 | 1.52 |
| Duration of trip | 0.71 | 1.42 | **-2.35*** |
| | | Alpha Amylase | |
| Disruptions in sleep | -0.96 | 1.73 | -1.87 |
| Time from awakening to assessment | 0.97 | -0.32 | 0.13 |
| Time from breakfast to assessment | -0.60 | -0.76 | 0.72 |
| Medication | 0.77 | **-1.93*** | 1.81 |
| Duration of trip | -0.20 | -1.41 | 1.53 |

* p<0.05 ** p < 0.01 *** p < 0.001

Given the central role played by A-A in the digestion of food (Granger et al, in press) it was tested if "time from breakfast to assessment" was the primary variable that accounted for the effects of other factors (except medication use, which logically, bears no relation with feeding) among that were related to A-A levels in the univariate analyses. Thus, sleep-related factors (namely "disrupted sleep" and "time from awakening to assessment"), "duration of trip" and "time from breakfast to assessment" were entered into another HMLM analysis. As expected, none of these factors were independently predictive of SAM activation once time from breakfast was controlled. The hypothesis that "time from breakfast to assessment" was the strongest predictor was supported since the other factors did not add extra predictive power once this factor had been accounted for ($\chi^2$ (9) = 8.57; $p$ > 0.50).

To sum up, "time from awakening to assessment" and "duration of trip" can be considered as the most significant child's state factors which were found to predict cortisol levels whereas "medication" and "time from breakfast to assessment" were the strongest predictors of A-A levels.

*The impact of technical factors*

In order to test which technical factors were able to predict cortisol and A-A levels, separate HMLM analyses were conducted. No technical factors, namely "cortisol kit" (old versus new kit), "cortisol assay" (duplicate versus single assay) and "methods of saliva collection" (rope versus sorbette), predicted cortisol response as shown in table 2.9. "Methods of saliva collection" had also no significant effect on salivary A-A response (table 2.9).

Table 2.9

*Technical factors as predictors of cortisol and A-A reactivity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| Cortisol Kits | 0.23 | 0.21 | -0.22 |
| Cortisol Assay | -0.73 | 0.43 | -0.64 |
| Methods of saliva collection | -1.02 | 0.39 | -0.28 |
| | | Alpha Amylase | |
| Methods of saliva collection | 0.40 | -0.94 | 1.26 |

## Discussion

The present study investigated the impact of some potentially interfering factors on salivary cortisol and alpha amylase reactivity in response to a stressor, the Strange Situation Procedure. Although the influence of some interfering factors was controlled when the study protocol had been designed (i.e. the choice of assessing infants in the morning to minimize cortisol circadian rhythm), their potential effects on physiological assessment needed to be evaluated. Indeed, the main findings of this investigation suggest the presence of some affecting factors on cortisol and alpha amylase assessment which should not be neglected in studies of psychobiology in infancy.

### *The impact of potential interfering factors on salivary cortisol assessment*

Salivary cortisol concentrations did not change from pre to post stressor in the total sample thus supporting other reports suggesting that it is difficult for many stressors to trigger a reaction of the HPA axis by the end of the first year of life (Gunnar et al., 1996; Gunnar and Donzella, 2002; Larson et al., 1998; Ramsay and Lewis, 1994). However, over 57% of infants showed an increase of cortisol levels post-stressor equal or above 10% from baseline values when an individual differences approach was considered. Although this finding was not statistically significant, the importance of investigating which factors can contribute to generate individual differences in glucorticoids response is suggested.

Socio-demographic, child's state as well as technical factors were examined as potential interfering variables with cortisol reactivity, as all of them have been indicated as potentially influential in previous research. As far as socio-demographic characteristics are concerned, none of them were associated with cortisol reactivity in line with several studies (Goldberg et al., 2003; Schieche and Spangler, 2005; van Bakel et al., 2004), with the exception of birth order. Surprisingly, first-born infants showed a significant decrease of cortisol concentrations post-stressor in comparison with later born infants who, conversely, showed an increase of cortisol values. This finding is not easy to interpret and might depend on the effects of other factors that were not measured in this study; consequently, further studies with larger samples are needed in order to confirm and better understand this data.

The findings of the present investigation on those child's state factors which could potentially be involved in HPA functioning provide, in general, support to previous empirical studies. Specifically, time from awakening to assessment, reflecting cortisol diurnal rhythm, and a specific situational factor, namely the duration of car trip to arrive to the laboratory, were found to significantly affect cortisol response more than all the other investigated variables.

In fact, infants who were assessed in a shorter time period from awakening had higher cortisol baseline levels than infants who were assessed later, and showed a significantly more rapid decrease of cortisol levels from pre to post SS after 20 min from the stressor and a subsequent recovery afterwards in comparison with the latter. The manifestation of these differences in cortisol response pattern between the two infants' groups is likely to be a sign of the diurnal decline of the HPA axis glucorticoid hormone. The data may suggest that cortisol declines relatively sharply over the morning hours even in infancy in contrast with previous studies which reported evidence of cortisol diurnal variation from midmorning to mid afternoon in this developmental age (e.g. De Weerth and van Geert, 2002; Gunnar and Donzella, 2002). Consequently, despite the assessment of glucorticoids response in the morning is a strategy commonly used by researchers in order to check for cortisol diurnal rhythm (e.g. Spangler and Schieche, 1998; van Bakel and Riksen-Walraven, 2004), our data suggest the importance of bearing in mind the presence, since morning, of a considerable variability in levels of cortisol baseline and reactivity in infants aged 12 to 18 months who are assessed in different time from awakening.

Furthermore, the duration of car trip to arrive to the laboratory was found to affect HPA axis functioning in line with other studies (Larson et al., 1991). Infants who had had a longer car trip showed a decrease of cortisol levels both at 20 and 40 min after the SS, whereas infants who had had a shorter car trip showed a rapid decrease at 20 min from the stressor followed by a rapid increase 40 min after it. Therefore, it can be inferred that the car ride produces a reduction of cortisol secretion as a consequence of its calming effects on infants as previously reported by Larson and her colleagues (1991). The condition of sleeping during the car trip, and also the duration of sleep during the trip were not associated with the inhibition of cortisol secretion. Similarly, the other sleeping- related factors (having disruptions in sleep and sleeping more the night before the assessment) which were investigated as potentially interfering conditions did not affect the production of this hormone.

In line with other studies (Gunnar et al., 1985; Spangler et al., 1991), feeding behaviour was found to be apparently associated with a deactivation of HPA axis functioning in terms of a decrease of cortisol concentrations in infants who were assessed after a shorter time from breakfast. However, the significant effect of feeding latency vanished when this variable was entered into the HMLM analyses along with other significant predictors. Similarly, the condition of taking medications, which was found to inhibit adrenocortical activity, did not affect cortisol response independently by other factors. In other words, both conditions cannot be considered independent predictors of HPA axis functioning as their effects depended on the association with the two significant predictors (time from awakening to assessment and the duration of car trip).

Last but not least, different methods of saliva collection, cortisol assay and kits were not found to interfere on HPA system. Amongst these methodological factors, the use of stimulants and sorbettes whenever infants refused to chew the cotton roll could represent a serious limitation of the current study in the light of findings from recent studies (Schwartz et al., 1998; Talge et al., 2005). In fact, Schwartz et al. (1998) highlighted the significance of the employment of stimulants, as they were found to interfere with some cortisol assays, while Talge et al. (2005) showed that oral stimulants increased or decreased, depending on which assay was used, the cortisol levels reported, therefore arguing against mixing methods of saliva collection. Although it is undoubtedly preferable to use just one method of saliva collection, the very little quantity of Kool Aid (not exceeding two or three grains) used in the present study is very likely to have had no effect on physiological assay.

*The impact of potential interfering factors on salivary alpha amylase assessment*

In contrast with HPA system, the SAM system was activated by the Strange Situation procedure in this study. Salivary alpha amylase levels significantly increased from pre to post stressor, as well as more than 70% of infants showed alpha amylase reactivity after the stressful procedure. Thus, further evidence to the significant role played by this parameter as a significant index of the physiological stress response in infancy is here supported (Granger et al, in press). However, another possible explanation of this data which cannot be neglected is that related to the diurnal variation of alpha amylase levels, recently described by Nater et al. (2007). In this study, significant changes were observed over the day with a pronounced decrease in the first 30 minutes after awakening, and gradually rising levels during the course of the day. Therefore, the progressive increases in alpha amylase levels from baseline to 20 and 40 minutes after the stressor found in the current study might reflect a diurnal pattern instead of SAM reactivity to stress. On the other hand, "time from awakening to assessment", likely to reproduce alpha amylase diurnal rhythm, did not independently predict the activity of the enzyme when other factors were taken into account in the HMLM analyses. To address this dilemma directly, it would be necessary to employ a control group in future study designs.

As far as we know, this is the first study which has investigated potential interfering factors on alpha amylase activity in response to a stressor in infancy. No socio-demographic and technical factors were likely to affect alpha amylase data, whereas two child's state factors, namely feeding latency and use of medication, interfere with alpha amylase response to the stress of separation.

The significant effect of feeding latency on the activity of alpha amylase was expected, considering that the digestion of macromolecules as carbohydrates is a primary function of this enzyme (Granger et al., in press). In fact, we found that infants who were assessed in a shorter time from breakfast had higher alpha amylase reactivity than infants who were assessed after a longer time. Therefore, it is likely that the recent consumption of food lead to increases in alpha amylase reactivity and further studies should take into account and control for this factor.

Similarly, data concerning the positive association between medication and alpha amylase response to stress was not unexpected. Although no studies had previously investigated the influence of medication on alpha amylase activity, Granger and colleagues (In press) suggest the potential importance of avoiding any prescription or OTC medication potentially interfering with the functioning of the para- or sympathetic nervous system. However, the small number of participants who took medication in the present study did not allow to identify which kind of medications had more impact on SAM activity. Therefore, further studies with larger samples can help to better clarify the role played by different medications in affecting alpha amylase activity in infancy.

In conclusion, salivary cortisol and A-A levels as indices of HPA axis and SNS functioning are likely to respond in a different way to a stressful laboratory procedure, namely the Strange Situation Procedure, in infancy. In fact, infants' physiological stress response was more marked for alpha amylase than for cortisol;

moreover, the two measures were not significantly correlated at any time. Last, different factors appear to affect cortisol and A-A baseline and reactivity levels, and should be taken into account in these kinds of studies. Specifically, factors concerning birth order, time from awakening to assessment, and duration of car trip will be considered as covariates when cortisol data are analyzed, whereas time from breakfast to assessment and medication will be considered as covariates when alpha amylase data are analyzed in the present study. In light of all these results, these measures seem to be two different indicators of the stress response in infants and, consequently, they should be both included in psychobiological studies in order to have a more complete understanding of physiological stress reactivity(Granger and Kivlighan, 2003).

# CHAPTER 3

# THE ROLE PLAYED BY THE ATTACHMENT RELATIONSHIP IN THE STRESS RESPONSE

## Introduction

Of the various social influences that mammals experience, caregivers are arguably the most powerful sources of stress and the most effective defence against harmful stressors. Disruption of the mother-infant relationship or failure of the caregiver to provide adequate care is likely to contribute to individual differences in physiological and behavioural responses to environmental challenges.

In the following paragraphs, after a brief synthesis concerning studies which have investigated effects of maternal care on stress response in animals and humans, the role played by child-mother attachment relationship in explaining individual differences in stress response will be reviewed.

### *Maternal care as a protective factor against stress*

#### Studies in animals

Research using animal models has shown how in many mammalian species early experiences shape the neurobiological systems involved in stress reactivity and regulation, and some of these effects appear permanent. The rat has been the focus of much of this research (Sanchez et al., 2001). Deprivation of maternal care during the relative stress hyporesponsive period (SHRP) in the rat, which is assumed to have evolved for protecting the developing brain from the potentially deleterious effects of elevated glucorticoids (GCs) and other neurochemicals (de Kloet et al., 1988), provoke marked activation of the hypothalamic-pituitary-adrenocortical (HPA) system and elevated brain levels of corticotropin releasing hormone (CRH) (Suchecki et al., 1993). However, if during this time maternal stimulation is mimicked, HPA and central (brain) CRH responses are controlled (Cirulli and Alleva 2003). Further, also normal variations in rat mothering impact the developing neurobiology of stress (Meaney and Szyf, 2005). In comparison with low-licking and -grooming dams, highlicking and -grooming dams have pups that, as adults, are less fearful and better able to contain and terminate stress reactions of the HPA axis (Caldji et al., 1998). In fact, maternal licking and grooming regulates the extent to which glucorticoid receptor (GR) genes in the hippocampus become methylated (Weaver et al., 2001), particularly during the first week of the life in the rat. Methylation effectively silences genes. Licking and grooming reduce methylation of hippocampal GR genes in rats and GR genes determine how many hippocampal glucocorticoid receptors an animal will have. Consequently, because hippocampal GRs are involved in terminating the stress responses of the HPA system, low levels of hippocampal GRs mean poor or sluggish regulation, more prolonged stress reactions, and vulnerability to allostatic load over the animal's lifetime (Meaney and Szyf 2005, Weaver et al., 2001). These epigenetic effects of maternal care are potentially irreversible, except through pharmacological manipulations that induce widespread demethylation (Weaver et al., 2005).

The impact of early social stimulation becomes obvious when typical caregiving patterns are severely disrupted (Cirulli and Alleva 2003, Sanchez et al., 2001). Relevant findings include evidence that separated animals, compared with control and handled animals, exhibit larger airpuff startle responses, greater freezing and anxiety behaviours to cat odor, and two- to threefold greater adrenocorticotropic hormone (ACTH) and glucocorticoid responses to restraint stress as adults (Cirulli and Alleva 2003). Interestingly, some effects of maternal separation appear to be responsive to post-infancy modification as reported by Francis and colleagues (2002) who found that environmental enrichment reverses the effects of maternal separation on both HPA and behavioural responses to stress, with no effect on CRF mRNA expression.

Studies of nonhuman primates also demonstrate that poor rearing conditions and conditions that disrupt responsive maternal care affect the neural substrates of stress vulnerability and resilience as well as can have long-term impacts on the neurobiology of stress and negative emotionality (Sanchez et al., 2001). Separations from the attachment figure provokes acute behavioural distress and increases activity of the HPA and sympathoadrenergic-adrenomedulla (SAM) systems; interestingly, such stress reactions can be attenuated by surrogate caregivers during the separation (Levine and Wiener, 1988). Further, early neglectful maternal care can also produce offspring who as adults are more fearful, low in dominance, high in brain levels of CRH, and who exhibit persistent alterations in somatostatin and metabolites of serotonin, dopamine, and NE (Coplan et al., 1996, Rosenblum and Andrews 1994, Rosenblum et al., 1994). However, the long-term effects of social deprivation on the HPA axis are unclear (Mason, 2000). For example, unlike in the rat, no one has yet to demonstrate changes in hippocampal GR. Rather, the levels of stress neurobiology that are disturbed appear to involve the cortico-limbic circuits that evaluate and regulate responses to psychosocial threat, circuits that are still rapidly developing after birth in the monkey as they are in the human child.

Studies in humans

The development and organization of the stress-response systems may depend partly on the interactive behaviours of caregivers. By their sensitive responses to the child's states and signals, parents are able to reduce threats and stressful states in their children. From a behavioural perspective, with age infants are increasingly able to maintain their emotional organization independently of their caregivers. In the newborn stage, most infants become emotionally and physiologically affected by relatively harmless stimuli (e.g. sounds from the environment, see Brazelton, 1984) or events (e.g. measuring and weighing, Gunnar et al., 1989). In contrast, older infants are often able to independently organize their behaviour to a certain degree (referred to as synactive behavioural organization; Als, 1986) in many everyday situations, and must rely on the caregivers' assistance only in specific situations later on. Whenever the behavioural organization is threatened by a more or less strong disturbance or stressor, the infant may become disorganized. Depending on the infant's ability to maintain his or her behavioural organization, the caregiver's behaviour may or may not contribute to the reorganization. Spangler and colleagues (1994) provided empirical support for the regulatory function of maternal behaviour for the psychobiological organization of infants. In their study, 41 infants and their mothers were observed during play at 3, 6 and 9 months of age. The authors found specific patterns of associations between maternal sensitivity and the infant's negative emotional expression. Furthermore, significant associations were found between maternal behaviour and changes in adrenocortical activity at 3 and 6 months of age. Infants with highly insensitive mothers exhibited a heightened adrenocortical activity as compared to infants with at least moderately sensitive mothers. In their conclusions the authors claim that in the early months, when the child is not capable of autonomous behavioural regulation, even in low stress situations, regulation through maternal behaviour was observable both on the behavioural and on the physiological level.

Similarly, studies which have investigated SAM activity in infants showed how individual differences in salivary alpha-amylase activity are associated (or attuned) between mothers and infants. In a series of tasks designed to illicit emotional distress in 6-10 months infants (e.g. arm restraint), and indirectly in their mothers who were asked to watch without intervening, a positive correlation between mother's and infant's salivary alpha-amylase levels was found despite alpha amylase levels were higher for mothers than for infants in response to the challenge tasks (Kivlighan et al., 2005). Moreover, a positive correlation between maternal and infant salivary alpha amylase levels was found in a study which investigated the impact of the mothers' depression/anxiety on response to an acute stressor (Shea et al., 2006).

To sum up, infants' cortisol and alpha amylase levels in response to stress appear to be influenced by the nature of their social relationships with caregivers. After the first year, attachment security or the expectations of the availability of a sensitive parent in times of stress is thought to gain importance as an "internalized" coping mechanism in potentially threatening situations.

*Attachment patterns and stress reactivity*

An attachment relationship is formed with the caregiver in infancy unless there is no stable interactive presence, such as is the case in certain kinds of institutional rearing. All other infants, even those who are mistreated, display attachment behaviours in terms of using the caregiver as a source of comfort and reassurance in the face of challenges or threats from the environment. Thus, child-mother attachment relationship is thought to be one of the most powerful strategies the child has to cope with stressful circumstances and negative emotions in an organized manner. However, the nature of the affective tie and the effectiveness with which the caregiver can be used as a source of comfort in the face of danger appears to differ across infant-caregiver dyads. Individual differences in the quality of attachment relationships are supposed to depend on the dyad's history and can be assessed by the Strange Situation (Ainsworth et al., 1978) which yields a classification of the infant's behavioural strategies with respect to attachment security (e.g. Ainsworth et al, 1978) and attachment disorganization (Main & Solomon, 1990). With respect to attachment security, infants can be classified as secure (B), insecure-avoidant (A), and insecure-ambivalent (C). These groups differ with respect to their ability to use the caregiver as a secure base for exploration, to express emotional concern after separation and to re-establish their emotional stability by seeking proximity and bodily contact with the attachment figure. Infants with secure attachment relationships are confident in the sensitive and responsive availability of their caregivers, and consequently these infants are confident in their own interactions with the world. On the contrary, infants who have not experienced consistent availability of and comfort from their caregivers or have experienced a consistently unavailable or rejecting parent when the environment has proven threatening will be unsure about the caregiver's availability as a "secure base". As a result they will be less prone to explore the environment and they will show more anxiety (anxious-resistant attachment) or will develop an apparent deactivation of attachment behaviours (avoidant attachment) during the Strange Situation. However, all these three attachment patterns are considered to imply the use of "organized" strategies which the infants carry out in order to establish a maximum of proximity given the infant's expectations of the caregiver's likely response (Main and Solomon, 1990). In contrast, disorganized attachment has been described as the breakdown of any consistent and organized strategy of emotion regulation during separation and reunion. Indeed, their behaviour is characterized by definite episodes of disorganization, like dazing, stilling, stereotypes, and/or contradictory behaviour patterns. Disorganized attachment behaviours are considered to reflect profound dysregulation of emotion because the caregiver represents simultaneously the source of fear as well as the only potential haven of safety (Van IJzendoorn et al., 1999).

In the light of these different behavioural strategies to cope with stress displayed by infants with different attachment patterns, some researchers have started to investigate if individual differences in the child-mother attachment relationship are also related to different physiological responses to the Strange Situation and other stressful procedures. Two possible models, the coping and the distress model, have been used to interpret these individual differences (e.g. Spangler and Schieche, 1998). The general idea incorporated in the coping model is that physiological activation can be most prominent in situations in which behavioural coping responses are not applicable or available, as might be argued to be the case in insecure attachment relationships. In contrast to this model, the distress model proposes that individual differences in stress response can be related to infant's predisposition to behavioural distress rather than to availability of behavioural strategies to cope with stressful situations. In other words, infants who are more prone to behavioural distress might be at higher risk to exhibit cortisol and alpha amylase reactivity than infants who are not. As the way of showing distress during the Strange Situation differ between infants classified as insecure (A versus C) as well as between infants classified as secure (B1-B2 versus B3-B4), the traditional attachment classification can be reviewed by combining infants into groups with low (A, B1,B2) and high proneness (B3,B4,C) to distress (Belsky and Rovine, 1987; Frodi and Thompson, 1985).

Most of the studies have examined physiological reactivity to stress in infants with different attachment status mainly focusing on the activation of the HPA axis as indexed by salivary cortisol (Gunnar et al., 1989; Gunnar et al., 1996; Hertsgaard et al., 1995; Nachmias et al., 1996; Schieche and Spangler, 2005; Spangler and Grossman, 1993; Spangler and Schieche, 1998). Some of these studies found that attachment security was related to lessened cortisol reactivity in response to the Strange Situation while adrenocortical activation was observed in insecurely attached and disorganized infants (Hertsgaard et al., 1995; Spangler and Grossman, 1993; Spangler and Schieche, 1998). Moreover, the findings of the other studies suggested the function of a secure attachment relationship as a social buffer against inappropriate behavioural dispositions in different stressful situations (Gunnar et al., 1996; Nachmias et al., 1996; Spangler and Schieche, 1998; Schieche and Spangler, 2005); specifically, behavioural inhibition was found to be associated with adrenocortical activation in response to stress only in insecurely attached infants. To our knowledge, just two studies have tested the distress model using the Belsky and Rovine (1987) classification of attachment patterns. However, researchers who have investigated HPA functioning in infants combined into groups with low and high proneness to distress did not find differences in cortisol reactivity following the strange situation (Spangler and Grossman, 1993; Spangler and Schieche, 1998).

A smaller number of studies have investigated indices of SAM activity in relation to infants' attachment status (Spangler and Grossman, 1993; Sroufe and Waters, 1977; Zelenko et al., 2005). These studies have measured changes in cardiac activity as indicators of emotional arousal or activation even in the absence of behavioural indicators of stress. Indeed, heart activity may be an index of a fast-acting and short-lasting activation to stressful situations, and the heart response is not subject to behavioural coping mechanisms (Ursin et al., 1978). The first of these studies was carried out by Sroufe and Waters (1977) and reported how in spite of the lack of overt distress avoidant children experience tachycardia during separation, which is similar to other attachment types, and fail to show the deceleration after reunion with mother that is typical of secure children. Spangler and Grossman (1993) also found that heart rate increases during the separation from the mother in all attachment groups, including the avoidant infants. Disorganized infants exhibited a particularly high heart rate elevation. A recent study replicated the finding that tachycardia occurs during separation in insecure-avoidant children in spite of a lack of overt signs of distress, but did not find any difference between attachment styles with respect to hearth deceleration after reunion between child and parent (Zelenko et al., 2005). Last, just one study has measured salivary alpha amylase in 12 months infants in response to the strange situation (Hill et al., 2006). Although on average the change in salivary alpha amylase in response to the strange situation was not significant, significant differences emerged in children classified as "avoidant" versus "secure" in the direction of higher alpha amylase levels in avoidant infants in comparison with those of securely attached infants. Last, no studies have investigated SAM reactivity in infants grouped according to Belsky and Rovine (1987) classification.

The main objective of this study was to evaluate the bio-behavioural organization of infants with different quality of attachment relationship. From the perspective of attachment theory, cortisol and alpha amylase increases were expected in Avoidant and Resistant infants who are thought to have suboptimal coping strategies and in Disorganized infants who are thought to demonstrate a lack of coherent strategy for dealing with separation. In contrast, no cortisol and alpha amylase response or at least a smaller increase, were expected in securely attached infants exhibiting an adequate behavioural strategy by re-establishing contact with their attachment figure. Moreover, from a different perspective based on distress model it was hypothesized that infants classified as B3, B4, C1 and C2 - who show intense separation distress and prolonged recovery during the Strange Situation -

would have exhibited higher cortisol and alpha amylase reactivity in response to stress than infants classified as A1, A2, B1 and B2. Last, an approach based on the global ratings to assess ABC attachment patterns ("proximity seeking", "contact maintaining", "avoidance of the mother" and "resistance to comforting" scales) as well as the total score on the Disorganized Attachment Scale was also used.

## Methods

The main features of sample's composition, procedure, salivary cortisol and alpha amylase collection and assay are summarised below. Full details about these methodological characteristics are reported in chapter 2.

### *Participants*

The initial sample consisted of 82 health infants (45 boys and 37 girls) aged 12 to 18 months (mean age = 14.6; SD = 1.8) and their mothers who gave their written consent for participating in the study. Most of the children's families represent middle-high socioeconomic status, including 47,5% upper middle class, 45% middle class, and 7,5% lower class as assessed by Hollingshead classification (1975). As already described in chapter 2, 8 (9,7%) and 6 (7,3%) infants were not included in the present analyses because they did not have any cortisol and alpha amylase data respectively. The final sample included 74 and 76 infants with cortisol and alpha amylase data respectively, whose 51 and 50 infants had all physiological measures and 49 infants had both completed cortisol and alpha amylase data. Infants of the three groups (all – partial – missing cortisol and alpha amylase data) did not differ on any of the study variables with the exception of "time of wake up" for cortisol data and "hours of sleep last night" and "disruptions in sleep" for alpha amylase data.

The assessment procedure took place in one morning session at the Psychopathology Unit of "Eugenio Medea" Scientific Institute, where the assay of salivary cortisol was also performed. Saliva was collected before the Strange Situation as well as 20 and 40 minutes after the procedure. Mothers also completed a brief socio-demographic form and provided information regarding those factors which can affect basal cortisol and alpha amylase levels.

*Instruments and psychophysiology assessment*

In order to collect socio-demographic variables, an ad hoc form to be filled out by parents was designed. To control for factors which can interfere with basal cortisol and alpha amylase assessment, information on time of assessment, the infant's health, mood, sleep, feeding, medications and duration of the trip to the lab were also recorded in an ad hoc form.

**The Strange Situation Procedure (SS).** The quality of the infant-mother attachment relationship was assessed using the Strange Situation (Ainsworth et al., 1978), which is a structured observational procedure consisting of seven 3-minute episodes, including two separations and two reunions with the caregiver in an unfamiliar setting.

Videotapes were evaluated for attachment security as described by Ainsworth, Blehar, Waters & Wall (1978) and for organized versus disorganized status as described by Main and Solomon (1990). The writer has been trained for the administration of the Strange Situation and its coding by Alan Sroufe and Elisabeth Carlson at the Institute of Child Development (University of Minnesota). The classification of attachment patterns are established from global ratings of infant's proximity seeking, contact maintaining, avoidance of the mother and resistance to comforting. *Secure* (B) infants communicate openly their emotions and are able to use the mother as a secure base for exploration. They freely explore the environment with occasional visual, verbal or physical contact when the mother is present but their exploration decrease when the mother departures. They may cry or not, but when she returns they greet her positively and, if they are upset, they go to her, are soon comforted and return to explore. *Avoidant* (A) infants explore with little reference to the mother, minimize the expression of negative emotions when she departures and, visibly, avoid contact with her upon reunions. *Anxious-Resistant* (C) infants are reluctant to explore even when the mother is present and are very distressed by her departures. They show ambivalence during the reunions episodes as they make strong efforts to make contact with the mother but they also resist her comforting efforts.

Within each of these patterns, Ainsworth identified 4 subgroups in the secure group (B1-B4), two in the avoidant group (A1,A2), and two in the resistant group (C1,C2). In the secure group, B1 and B2 infants are somewhat like avoidant infants in that they showed minimal upset and displayed less contact-seeking during reunions than B3 and B4 infants. Infants classified as B3 and B4 are somewhat like resistant infants in being readily upset by separations and engaging in strong contact seeking at reunions. In the avoidant group, infants classified as A1 are consistently avoidant whereas infants classified as A2 show mixed behaviour with some tendency to approach mixed with avoidance of the mother. In the resistant group, infants classified as C1 are overtly angry while infants classified as C2 are more passive and helpless.

In contrast to secure, avoidant and resistant infants who exhibit organized strategies for relating to the caregiver when distressed, *disorganized* (D) infants are unable to maintain a consistent strategy because of the breakdown of their preferred ones. Consequently to this failure, the infant's behaviour becomes unusual and unpredictable. The coding system for disorganized attachment encompasses seven categories of behaviour: 1) Sequential display of contradictory behaviour patterns (e.g. in the middle of a display of anger and distress, the infant suddenly becomes markedly devoid of affect and moves away from the parent); 2) Simultaneous display of contradictory behaviour patterns (e.g. infant approaches by backing back toward parent); 3) Undirected, misdirected, incomplete, and interrupted movements and expressions (e.g. infant cries at stranger's leavetaking, attempts to follow her out of the room); 4) Stereotypes, asymmetrical movements, mistimed movements and anomalous postures (e.g. Extended rocking, ear pulling, hair twisting and any other rhythmical, repeated movements without visible function); 5) Freezing, stilling and slowed movements and expressions (e.g. Freezing lasting 20 seconds or more, and stilling lasting 30 seconds or more, accompanied by dazed or trance-like facial expression); 6) Direct indices of apprehension regarding the parent (e.g. fearful facial expression on pick up); 7) Direct indices of disorganization or disorientation (e.g. "Greeting" stranger brightly at the moment of reunion with parent). Given that infants differ in the intensity, context and frequency of their display of disorganized-disoriented behaviours, each infant is rated on a 9-point scale for D behaviours and those with a D score >= 5 are classified as disorganized. If an infant is thought to fit the disorganized category best, an effort is also made to determine the underlying strategy (secure, avoidant or resistant). Just in case no underlying strategy may be identified, it can be used the category "unclassifiable" followed by a guess (e.g. disorganized-unclassifiable-avoidant).

For the purposes of the interrater reliability, 35% of the tapes (N = 29) were also coded by another psychologist of the Scientific Institute (IRCCS) "Eugenio Medea" specifically trained for the traditional classification system ABC. Intercoder agreement was 82.7% (Cohen's Kappa = .74).

**Salivary cortisol and alpha amylase collection and assay.** The cortisol and alpha amylase response was assessed from saliva. To collect saliva, the infants were encouraged to suck/chew on sterile a 6-inch cotton dental roll lightly dusted on the tip with 2 or three grains of Kool Aid crystals. Once the tip of the roll was soaked with saliva, it was cut off and placed in a needleless syringe. The clear saliva was expressed into a cryogenic vial for storage at – 80° C. If the children did not cooperate in chewing on the cotton roll, a microsponge (Sorbette -Salimetrics), was placed in the mouth for around 1 minute to collect saliva; then, the plastic shaft of the Sorbette was cut off half and inserted into a 2mL cryovial with the absorbent end up for storage at – 80° C until being assayed. To extract the saliva from sorbettes, the cryovials were centrifuged for 20 minutes at 1500 Xg before the assays.

The assessment of cortisol levels was initially done by the Salimetrics HS-Cortisol EIA Kit and then by a new updating of the kit (Expanded Range (ER) HS-Cortisol EIA Kit, Salimetrics), two competitive immunoassays specifically designed for the quantitative measurement of salivary cortisol. The assessment took place in the biology lab of the IRCCS "Eugenio Medea". Cortisol values assayed with the previous kit were transformed according to the following formula: Expanded Range HS-cortisol = ((-0.02293) + (HS cortisol *0.954)) with the aim to compare findings obtained with the two kits. All samples from one infant were analyzed in one assay to minimize the variability of the results. To guarantee validity of analysis, duplicate assays were performed whenever possible. Average intra-and interassay coefficients of variation were less than 9% and 10% respectively.

Samples were assayed for alpha amylase (A-A) using a commercially available kinetic reaction assay (Salimetrics, State College, PA) in the Salimetrics lab (University of Pennsylvania). The test has a reported average inter assay variation computed for the mean of average duplicates for 8 separate runs for lower (10,6 U/mL) and higher (166.0 U/mL) concentrations samples were 5,8 and 3,6 % respectively whereas intrassay variation computed for the mean of 10 replicate tests of low (17,7% U/mL), medium (108,8 U/mL), and high (474,6 U/mL) concentrations samples were 7,2%, 6,7%, and 2,5%, respectively (Granger et al, in press).

Previous analyses (see chapter 2) revealed no significant effects of saliva collection methods (rope versus sorbette), cortisol assay (duplicate versus singlet) and cortisol kits on physiological measures.

*Statistical analyses*

In order to check eventual associations which could confound findings, preliminary chi-square analyses between each socio-demographic and child's state factors and the four attachment groups were performed.

The effects of attachment patterns on stress reactivity were tested by hierarchical multivariate linear models (HMLM) analyses. HMLM analyses, including parameters for intercept as well as linear and quadratic change, were executed separately for salivary cortisol as the outcome variable, and 1) attachment patterns; 2) secure versus insecure attachment; 3) attachment classification coded in terms of A1-B2 versus B3-C2; and 4) attachment scales as the predictor variables. The following variables "birth order", "time from awakening to assessment", and "duration of car trip" were also entered into the model as they were found to predict cortisol reactivity (see chapter 2). Separate HMLM analyses were also used for salivary alpha amylase as the outcome variable, and 1) attachment patterns; 2) secure versus insecure attachment; 3) attachment classification coded in terms of A1-B2 versus B3-C2; and 4) attachment scales as the predictor variable. "Time from breakfast to assessment" and "medication" which were found to predict alpha amylase reactivity (see chapter 2) were also entered into the model. A more detailed description of the HMLM analyses is reported in chapter 2.

## Results

*Preliminary Analyses: attachment and potential confounds*

In the original sample, attachment patterns were distributed as follows: 43 secure (52,4%), 10 avoidant (12,2%), 14 anxious-resistant (17,1%) and 15 disorganized (18,3%) infants. Attachment status did not differ according to any child's and family demographic characteristics, as well as according to any child's state factors – related to sleep, food, physical conditions and daily experiences –

which were investigated as potential interfereeing variables on stress response (see chapter 2). In the final sample, involving infants with cortisol data, 38 (51,4%) infants were classified as secure, 10 (7.4 %) infants were classified as avoidant, 12 (16.2%) infants were classified as anxious-resistant, and 14 (18.9%) (2 B; 9 A; 3 C) infants were classified as disorganized whereas in respect to infants with alpha amylase data 39 (51.3%) infants were classified as secure, 10 (7.6%) infants were classified as avoidant, 12 (15.8%) infants were classified as anxious-resistant, and 15 (19.7%) (2 B; 10 A; 3 C) were classified as disorganized. The distribution of attachment patterns among each of the two final samples did not differ from the original sample (cortisol data: $\chi^2$ (3) = 1,74 $p$ = 0.63; alpha amylase data: $\chi^2$ (3) = 3,23 $p$ = 0,36). In tables 3.1 and 3.2 the distributions of attachment patterns according to socio-demographic characteristics are shown for the samples with cortisol and alpha amylase data respectively. No significant associations were found between attachment categories and each demographic characteristic investigated. Similarly, attachment status was not related to any of the child's state factors considered.

Table 3.1

*Distribution of the attachment patterns according to child's and family demographic characteristics in infants with cortisol data.*

| | ***ATTACHMENT PATTERN*** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***B*** | | ***A*** | | ***C*** | | ***D*** | | ***Statistical analyses*** | |
| | *f* | *%* | *f* | *%* | *f* | *%* | *F* | *%* | $\chi^2$ | *p* |
| **CHILD'S DEMOGRAPHIC CHARACTERISTICS** | | | | | | | | | | |
| **GENDER** | | | | | | | | | | |
| *Boys* | 21 | 55.3 | 5 | 50 | 6 | 50 | 8 | 57.1 | 0.22 | 0.97 |
| *Girls* | 17 | 44.7 | 5 | 50 | 6 | 50 | 6 | 42.9 | | |
| **AGE** | | | | | | | | | | |
| *12-14 months* | 17 | 44.7 | 6 | 60 | 8 | 66.7 | 5 | 35.7 | 3.25 | 0.36 |
| *15-18 months* | 21 | 55.3 | 4 | 40 | 4 | 33.3 | 9 | 64.3 | | |
| **BIRTH ORDER** | | | | | | | | | | |
| *First born* | 25 | 65.8 | 7 | 70 | 8 | 66.7 | 10 | 71.4 | .18 | 0.98 |
| *Second born or third born* | 13 | 34.2 | 3 | 30 | 4 | 33.3 | 4 | 28.6 | | |
| **FAMILY DEMOGRAPHIC CHARACTERISTICS** | | | | | | | | | | |
| **MATERNAL AGE** | | | | | | | | | | |
| *< 35 years* | 24 | 63.2 | 7 | 70 | 9 | 75 | 7 | 50 | 1.97 | 0.58 |
| *> 35 years* | 14 | 36.8 | 3 | 30 | 3 | 25 | 7 | 50 | | |
| **PATERNAL AGE** | | | | | | | | | | |
| *< 35 years* | 15 | 39.5 | 5 | 50 | 2 | 16.7 | 5 | 35.7 | 2.97 | 0.40 |
| *> 35 years* | 23 | 60.5 | 5 | 50 | 10 | 83.3 | 9 | 64.3 | | |
| **MATERNAL EDUCATION** | | | | | | | | | | |
| *Less than 10 years* | 5 | 13.2 | 1 | 10 | 2 | 16.7 | 4 | 30.8 | 2.54 | 0.47 |
| *More than 10 years* | 33 | 86.8 | 9 | 90 | 10 | 83.3 | 9 | 69.2 | | |
| **PATERNAL EDUCATION** | | | | | | | | | | |
| *Less than 10 years* | 5 | 13.2 | 4 | 40 | 4 | 33.3 | 4 | 30.8 | 4.83 | 0.19 |
| *More than 10 years* | 33 | 86.8 | 6 | 60 | 8 | 66.7 | 9 | 69.2 | | |
| **SES** | | | | | | | | | | |
| *Low* | 3 | 8.1 | 2 | 2.2 | 0 | 0 | 1 | 7.1 | 4.87 | 0.56 |
| *Medium* | 16 | 43.2 | 4 | 44.4 | 4 | 33.3 | 7 | 50 | | |
| *High* | 18 | 48.6 | 3 | 33.3 | 8 | 66.7 | 6 | 42.9 | | |
| **INCOME** | | | | | | | | | 1.08 | 0.78 |
| *From 10,000 – 31,000 Euros* | 13 | 37.1 | 5 | 55.6 | 4 | 44.4 | 5 | 38.5 | | |
| *More than 31,000 Euros* | 22 | 62.9 | 4 | 44.4 | 5 | 55.6 | 8 | 61.5 | | |

Table 3.2

*Distribution of the attachment patterns according to child's and family demographic characteristics in infants with alpha amylase data.*

| | *ATTACHMENT PATTERN* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***B*** | | ***A*** | | ***C*** | | ***D*** | | ***Statistical analyses*** | |
| | *f* | % | *f* | % | *f* | % | *f* | % | $\chi^2$ | *p* |
| **CHILD'S DEMOGRAPHIC CHARACTERISTICS** | | | | | | | | | | |
| **GENDER** | | | | | | | | | | |
| *Boys* | 21 | 53.8 | 5 | 50 | 6 | 50 | 9 | 60 | 0.36 | 0.95 |
| *Girls* | 18 | 46.2 | 5 | 50 | 6 | 50 | 6 | 40 | | |
| **AGE** | | | | | | | | | | |
| *12-14 months* | 17 | 43.6 | 6 | 60 | 8 | 66.7 | 5 | 33.3 | 3.84 | 0.28 |
| *15-18 months* | 22 | 56.4 | 4 | 40 | 4 | 33.3 | 10 | 66.7 | | |
| **BIRTH ORDER** | | | | | | | | | | |
| *First born* | 26 | 66.7 | 7 | 70 | 8 | 66.7 | 11 | 73.3 | 0.25 | 0.97 |
| *Second born or third born* | 13 | 33.3 | 3 | 30 | 4 | 33.3 | 4 | 26.7 | | |
| **FAMILY DEMOGRAPHIC CHARACTERISTICS** | | | | | | | | | | |
| **MATERNAL AGE** | | | | | | | | | | |
| *< 35 years* | 25 | 64.1 | 7 | 70 | 9 | 75 | 8 | 53.3 | 1.53 | 0.68 |
| *> 35 years* | 14 | 35.9 | 3 | 30 | 3 | 25 | 7 | 46.7 | | |
| **PATERNAL AGE** | | | | | | | | | | |
| *< 35 years* | 16 | 41 | 5 | 50 | 2 | 16.7 | 6 | 40 | 3.10 | 0.38 |
| *> 35 years* | 23 | 59 | 5 | 50 | 10 | 83.3 | 9 | 60 | | |
| **MATERNAL EDUCATION** | | | | | | | | | | |
| *Less than 10 years* | 5 | 12.8 | 1 | 10 | 2 | 16.7 | 4 | 28.6 | 2.21 | 0.53 |
| *More than 10 years* | 34 | 87.2 | 9 | 90 | 10 | 83.3 | 10 | 71.4 | | |
| **PATERNAL EDUCATION** | | | | | | | | | | |
| *Less than 10 years* | 5 | 12.8 | 4 | 40 | 4 | 33.3 | 4 | 28.6 | 4.93 | 0.18 |
| *More than 10 years* | 34 | 87.2 | 6 | 60 | 8 | 66.7 | 10 | 71.4 | | |
| **SES** | | | | | | | | | | |
| *Low* | 3 | 8.1 | 2 | 20 | 0 | 0 | 1 | 6.7 | 5.34 | 0.50 |
| *Medium* | 16 | 43.2 | 4 | 40 | 4 | 33.3 | 8 | 53.3 | | |
| *High* | 18 | 48.6 | 3 | 30 | 8 | 66.7 | 6 | 40 | | |
| **INCOME** | | | | | | | | | 1.32 | 0.73 |
| *From 10,000 – 31,000 Euros* | 13 | 36.1 | 5 | 55.6 | 4 | 44.4 | 5 | 35.7 | | |
| *More than 31,000 Euros* | 23 | 63.9 | 4 | 44.4 | 5 | 55.6 | 9 | 64.3 | | |

*Cortisol and alpha amylase reactivity according to attachment status*

Cortisol and alpha amylase log transformed mean and SD values before and after the Strange Situation shown by infants with different attachment styles are displayed in table 3.3. In order to test if attachment patterns predicted cortisol and alpha amylase levels, separate HMLM analyses were conducted. For cortisol, "birth order", "time from awakening to assessment", and "duration of trip" - which were found to significantly predict cortisol data (see chapter 2) - were entered into the model jointly with infant attachment classifications (B vs. A vs. C vs. D); similarly, for alpha amylase, "time from breakfast to assessment" and "medication" - which were found to significantly predict alpha amylase data (see chapter 2) - were entered into the model together with infant attachment classifications.

Table 3.3

*Cortisol and A-A mean and (SD) log transformed values by attachment status*

| | **Pre SS** | **Post 20 min** | **Post 40 min** |
|---|---|---|---|
| **Attachment Patterns** | **Cortisol (in µg/dl) reactivity** | | |
| Secure | -0,97 (0,29) | -1,00 (0,38) | -0,98 (0,23) |
| Avoidant | - 0,77 (0,29) | -1,04 (0,22) | -1,08 (0,31) |
| Resistant | -0,88 (0,15) | -0,86 (0,28) | -0,92 (0,23) |
| Disorganized | -0,87 (0,35) | -0,97 (0,23) | -0,97 (0,32) |
| *Total* | -0,92 (0,29) | -0,97 (0,33) | -0,98 (0,26) |
| | **A-A (in U/mL) reactivity** | | |
| Secure | 2.52 (0.36) | 2.55 (0.40) | 2.69 (0.31) |
| Avoidant | 2.59 (0.37) | 2.60 (0.41) | 2.78 (0.24) |
| Resistant | 2.29 (0.55) | 2.51 (0.60) | 2.55 (0.68) |
| Disorganized | 2.43 (0.39) | 2.62 (0.35) | 2.73 (0.29) |
| *Total* | 2.47 (0.41) | 2.56 (0.42) | 2.68 (0.38) |

As shown in table 3.4, "avoidant" attachment as well as "birth order", "time from awakening to assessment" and "duration of trip" significantly predicted cortisol concentrations, whereas "resistant" attachment as well as "time from breakfast to assessment" and "medication" significantly predicted alpha amylase concentrations. The direction of the effects related to attachment on cortisol and alpha amylase activity are displayed in figure 3.1.

Table 3.4

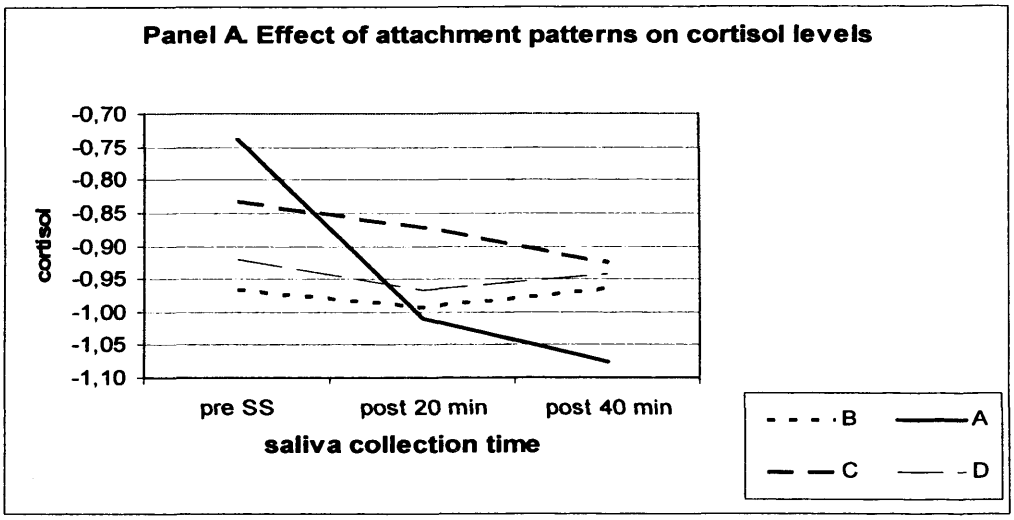
*Attachment patterns as predictors of cortisol and A-A activity. Results of the hierarchical linear model*

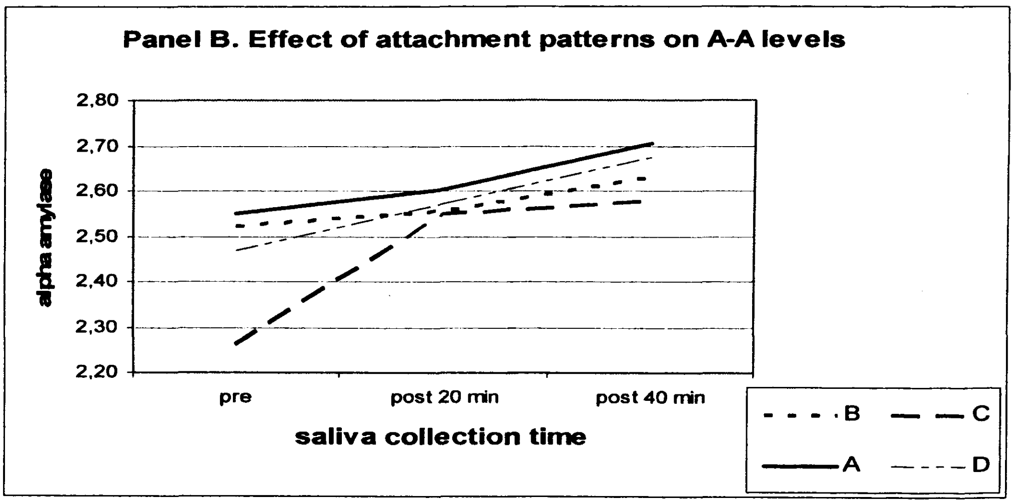
| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| **Avoidant** | **2.50**** | **-2.44**** | 1.65 |
| **Resistant** | 1.51 | 0.13 | -0.53 |
| **Disorganized** | 0.47 | -0.19 | 0.17 |
| Birth order | -0.48 | 2.01* | -1.39 |
| Time from awakening to assessment | -3.52*** | 3.84*** | -3.08** |
| Duration of the trip | 0.68 | 1.24 | -2.08* |
| | | Alpha Amylase | |
| **Avoidant** | 0.19 | 0.07 | 0.04 |
| **Resistant** | **-1.97*** | **2.36*** | -1.80 |
| **Disorganized** | -0.44 | 0.55 | -0.26 |
| Time from breakfast to assessment | 0.63 | -2.68** | 2.49** |
| Medication | 0.90 | -2.18* | 2.01* |

The primary variables of interest for this study (attachment classifications) are typed in bold, as well as their significant effect on cortisol data.
* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 3.1

*Significant effects of attachment patterns on salivary cortisol (inμg/dl ) and A-A (in U/mL ) log transformed concentrations*




As shown in panel A, infants with avoidant attachment demonstrated higher initial levels of cortisol and a larger decrease of cortisol from pre-Strange Situation to 20 and 40 minutes post-Strange Situation compared with infants with other attachment patterns; infants with anxious-resistant attachment showed a lower initial level of alpha amylase and a stronger increase in alpha amylase levels after the Strange Situation procedure compared to infants with other attachment patterns (panel B).

Infants were then combined into 2 attachment patterns (secure versus insecure) in order to test if security of attachment (B versus A,C,D) was a significant predictor of cortisol and alpha amylase activity, taking also into account those child's demographic and state factors which were found to predict HPA and SAM response. As shown in table 3.5, security of attachment did not predict the two physiological parameters, while "birth order", "time from awakening to assessment" and "duration of trip" did for cortisol levels, and "time from breakfast to assessment" and "medication" did for alpha amylase levels.

Table 3.5

*Attachment security (B VS A,C,D) as predictor of cortisol and A-A activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| **Secure Vs Insecure** | 1.89 $^{T}$ | -0.95 | 0.44 |
| Birth order | -0.57 | 2.10* | -1.48 |
| Time from awakening to assessment | -3.07** | 3.75*** | -3.16** |
| Duration of trip | 0.53 | 1.38 | -2.21* |
| | | Alpha Amylase | |
| **Secure Vs Insecure** | -1.14 | 1.57 | -1.10 |
| Time from breakfast to assessment | 0.49 | -2.46** | 2.27* |
| Medication | 0.72 | -1.94* | 1.81 $^{T}$ |

The primary variable of interest for this study (attachment security) is typed in bold.
* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

*Cortisol and alpha amylase reactivity according to behavioural distress (A1-B2 versus B3-C2)and attachment status*

In order to test whether attachment classification, when coded in terms of the A1-B2 versus B3-C2 framework, was a significant predictor of cortisol and alpha amylase activity, independently by attachment security and taking also into account child's demographic and state factors as covariates, two separate HMLM analyses were performed. Disorganized infants were excluded from these analyses.

As shown in table 3.6, cortisol levels were significantly predicted both by behavioural distress (A1-B2 versus B3-C2) and attachment security (secure versus insecure), whereas alpha amylase levels were not.

Table 3.6

*Cortisol and Alpha amylase levels predicted by A1-B2 versus B3-C2 attachment and secure versus insecure attachment groups. Results of the hierarchical linear model*

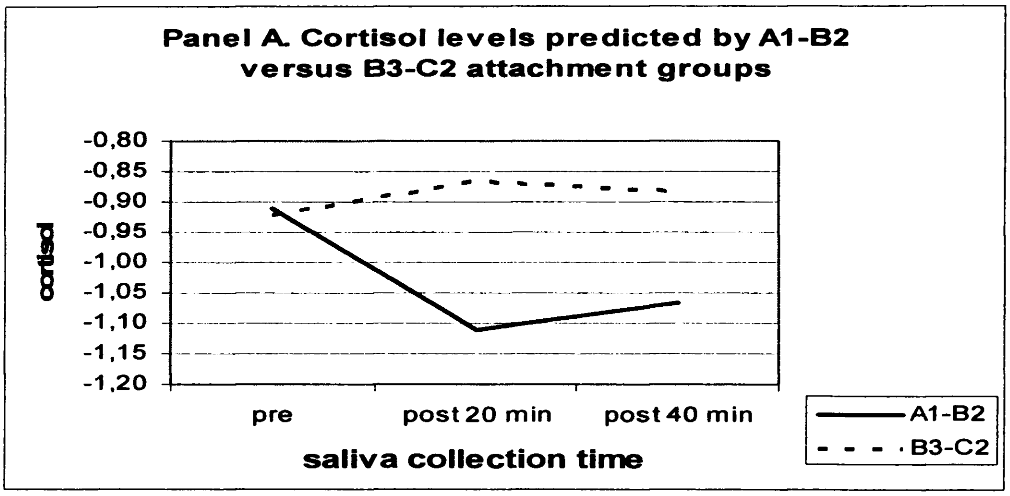
| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| **A1-B2 versus B3-C2** | -0.24 | **3.89***** | -3.19 |
| **Secure – Insecure** | **2.56**** | -1.34 | 0.53 |
| Birth order | -1.54 | 2.39* | -1.70 |
| Time from awakening to assessment | - 2.83** | 3.33** | -2.58** |
| Duration of trip | 0.58 | 2.11* | -2.55* |
| | | Alpha Amylase | |
| **A1-B2 versus B3-C2** | -0.99 | 1.37 | -1.12 |
| **Secure – Insecure** | -1.28 | 1.69 | -1.27 |
| Time from breakfast to assessment | 0.24 | -2.38* | 2.26* |
| Medication | 0.90 | -2.20* | 2.08* |

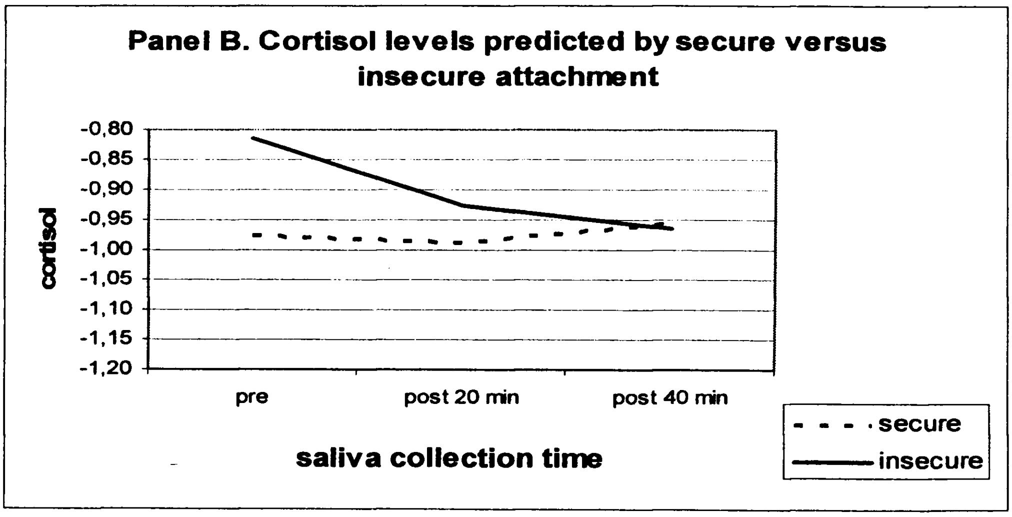
The primary variables of interest for this study (attachment groups) are typed in bold, as well as their significant effect on cortisol and alpha amylase data.
* p<0.05; ** p < 0.01; *** p < 0.001

Specifically, infants classified B3, B4, C1 and C2 in their attachment to mothers showed a slight increase of cortisol levels post the strange situation in comparison with infants classified A1, A2, B1 and B2 who showed a marked cortisol decreasing after the stressor (Figure 3.2; panel A). Moreover, infants classified as insecure showed higher cortisol baseline levels than secure infants (Figure 3.2; panel B).

Figure 3.2

*Significant effects of behavioural distress and attachment status on salivary cortisol concentrations*




*Cortisol and alpha amylase reactivity according to scales on attachment behaviours*

In order to test if global ratings of infants' "Proximity Seeking" (PS), "Contact Maintaining" (CM), "Resistance to Comforting" (Re) and "Avoidance of the Mother" (Av) – which are the four attachment scales for assessing ABC attachment patterns – as well as the "Total D scale" score for assessing disorganized attachment were significant predictors of salivary cortisol and alpha amylase activity, 18 exploratory HMLM analyses were performed with each attachment scale jointly with child's demographic and state factors found to predict stress response as the independent factors, and cortisol and alpha amylase as the outcome variable. As shown in table 3.7, all attachment scales (PS, CM, Re, Av) rated for the second reunion episode, as well as "Re" scale rated for the first reunion significantly predicted cortisol levels. "Re" scale rated for the second reunion was the only attachment scale which predicted alpha amylase levels (table 3.8). Scores on the "Total D scale" did not significant predict the response of the two physiological measures.

Table 3.7 *Attachment scales as predictors of cortisol activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Proximity Seeking V** | -0.65 | 1.28 | -1.27 |
| Birth order | -0.57 | 2.01* | -1.40 |
| Time from awakening to assessment | -3.07** | 3.78*** | -3.22** |
| Duration of trip | 0.52 | 1.41 | -2.21* |
| **Proximity Seeking VIII** | -0.78 | **2.58**** | **-2.27*** |
| Birth order | -0.61 | 2.06* | -1.41 |
| Time from awakening to assessment | -3.03** | 3.72*** | -3.10** |
| Duration of trip | 0.57 | 1.43 | -2.27* |
| **Contact Maintaining V** | 0.54 | 1.73 | -1.53 |
| Birth order | -0.57 | 1.89t | -1.28 |
| Time from awakening to assessment | -3.03** | 3.80*** | -3.20** |
| Duration of trip | 0.55 | 1.55 | -2.38* |
| **Contact Maintaining VIII** | -1.00 | **3.25**** | **-3.11**** |
| Birth order | -0.46 | 1.67 | -1.04 |
| Time from awakening to assessment | -3.03** | 3.73*** | -3.11** |
| Duration of trip | 0.54 | 1.56 | -2.39* |
| **Resistance V** | -0.14 | **2.09*** | **-2.13*** |
| Birth order | -0.68 | 2.28* | -1.65 |
| Time from awakening to assessment | -2.99** | 3.92*** | -3.34*** |
| Duration of trip | 0.64 | 1.11 | -1.96* |
| **Resistance VIII** | -0.40 | **3.48***** | **-3.51***** |
| Birth order | -0.67 | 2.43* | -1.76 |
| Time from awakening to assessment | -3.04** | 3.85*** | -3.27*** |
| Duration of trip | 0.58 | 1.47 | -2.35* |
| **Avoidance V** | 0.01 | -0.82 | 0.89 |
| Birth order | -0.65 | 2.12* | -1.50 |
| Time from awakening to assessment | -3.00** | 3.63*** | -3.07** |
| Duration of trip | 0.62 | 1.33 | -2.15** |
| **Avoidance VIII** | 1.61 | **-2.82**** | **2.44*** |
| Birth order | -0.56 | 2.17* | -1.51 |
| Time from awakening to assessment | -2.85** | 3.22** | -2.65** |
| Duration of trip | 0.36 | 1.70 | -2.45* |
| **D** | -0.84 | 1.01 | -0.74 |
| Birth order | -0.71 | 2.12* | -1.47 |
| Time from awakening to assessment | -3.12** | 3.90*** | -3.28** |
| Duration of trip | 0.74 | 1.17 | -2.07* |

The primary variables of interest for this study (attachment scales) are typed in bold, as well as their significant effect on cortisol data.
p<0.05; ** p < 0.01; *** p < 0.001

Table 3.8

*Attachment scales as predictors of alpha amylase activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Proximity Seeking V** | 0.25 | 0.86 | -0.56 |
| Time from breakfast to assessment | -0.75 | -1.93* | 1.88t |
| Medication | 0.72 | -1.29 | 1.20 |
| **Proximity Seeking VIII** | 0.72 | 0.12 | -0.16 |
| Time from breakfast to assessment | 0.45 | -2.40* | 2.22* |
| Medication | 0.67 | -1.98 | 1.86t |
| **Contact Maintaining V** | -1.63 | 1.50 | -1.05 |
| Time from breakfast to assessment | 0.49 | -2.49** | 2.32* |
| Medication | 0.61 | -1.88t | 1.78t |
| **Contact Maintaining VIII** | -1.75 | 1.57 | -1.22 |
| Time from breakfast to assessment | 0.60 | -2.54** | 2.33* |
| Medication | 0.61 | -1.87t | 1.77 |
| **Resistance V** | -0.82 | 1.60 | -1.31 |
| Time from breakfast to assessment | 0.47 | -2.42* | 2.24* |
| Medication | 0.69 | -1.89t | 1.78 |
| **Resistance VIII** | **-2.08*** | **2.12*** | -1.66 |
| Time from breakfast to assessment | 0.73 | -2.74** | 2.53** |
| Medication | 0.65 | -1.97* | 1.84t |
| **Avoidance V** | -0.42 | -1.59 | 1.16 |
| Time from breakfast to assessment | 0.39 | -2.72** | 2.51** |
| Medication | 0.73 | -2.10* | 1.92t |
| **Avoidance VIII** | -0.31 | -1.56 | 1.37 |
| Time from breakfast to assessment | -0.41 | -2.63** | 2.44* |
| Medication | 0.70 | -2.22* | 2.07* |
| **D** | 0.26 | -0.28 | 0.65 |
| Time from breakfast to assessment | 0.49 | -2.39* | 2.27* |
| Medication | 0.74 | -2.00* | 1.88t |

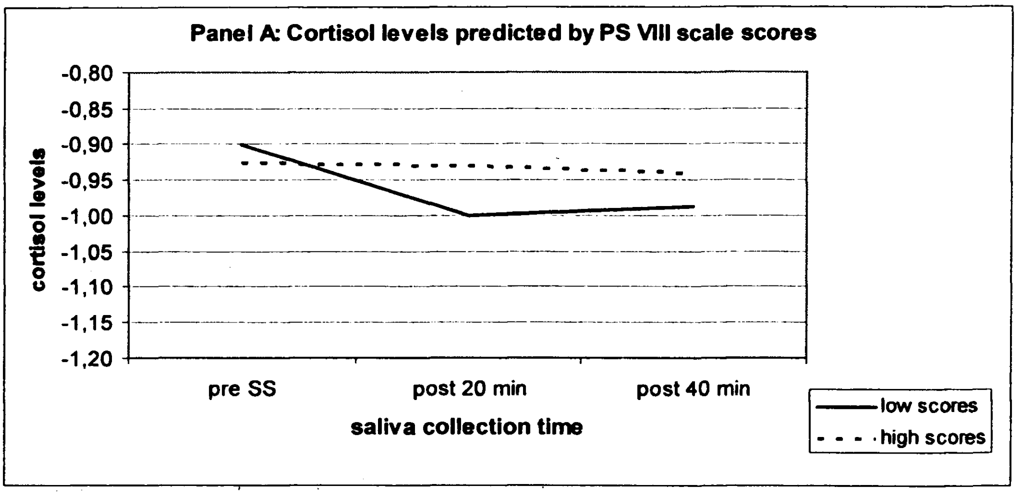
The primary variables of interest for this study (attachment scales) are typed in bold, as well as their significant effect on alpha amylase data.
* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

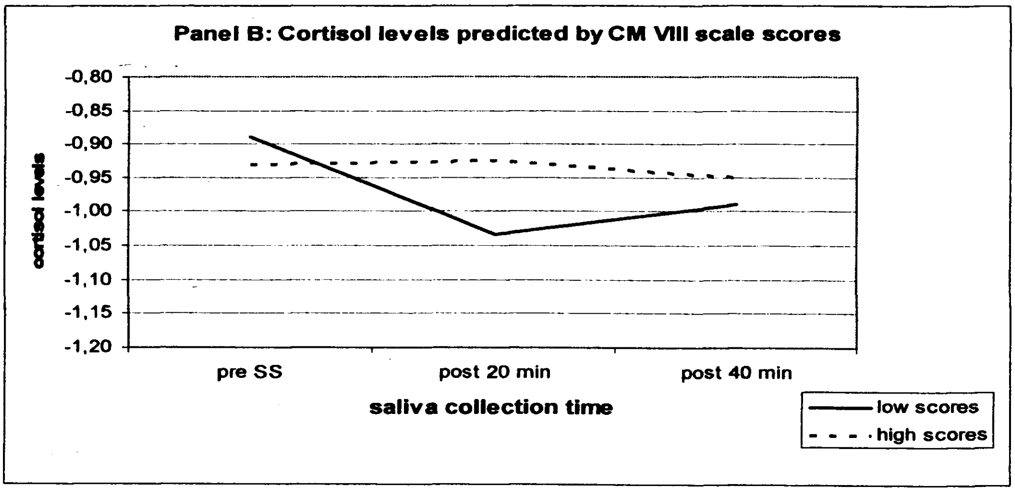
In figure 3.3 the direction of each significant effect on cortisol and alpha amylase response is shown. Specifically, infants with low scores on the "Proximity Seeking" scale had a more marked decrease of cortisol concentrations after the SS than infants with high scores on this scale, who actively searched proximity to the mother after the second separation (panel A). Infants with low scores on the "Contact Maintaining" scale showed a decrease of cortisol concentrations from pre to post the SS than infants with high scores on this scale, who actively tried to maintain contact with the mother after the second separation (panel B). Infants with low scores on the "Resistance to Comforting" scales rated for both first and second reunion, had cortisol decreasing from pre to 20 min post the SS followed by a slight increase at 40 min post the procedure than infants with high scores on these scales, who actively resisted to contact with the mother during the reunion (panel C and D). Last, infants with high scores on the "Avoidance of the Mother" scale showed a marked decrease of cortisol concentrations from pre to 20 min post the SS than infants with low scores on this scale, who did not avoid the mother during the second reunion (panel E). In relation to SAM activity, infants with high scores on the "Resistant to Contact" scale for the second reunion showed low alpha amylase baseline levels and a more marked increase of alpha amylase after the SS than infants with low scores on this scale, although these latter had the highest alpha amylase concentrations for all three saliva collection times (panel F).

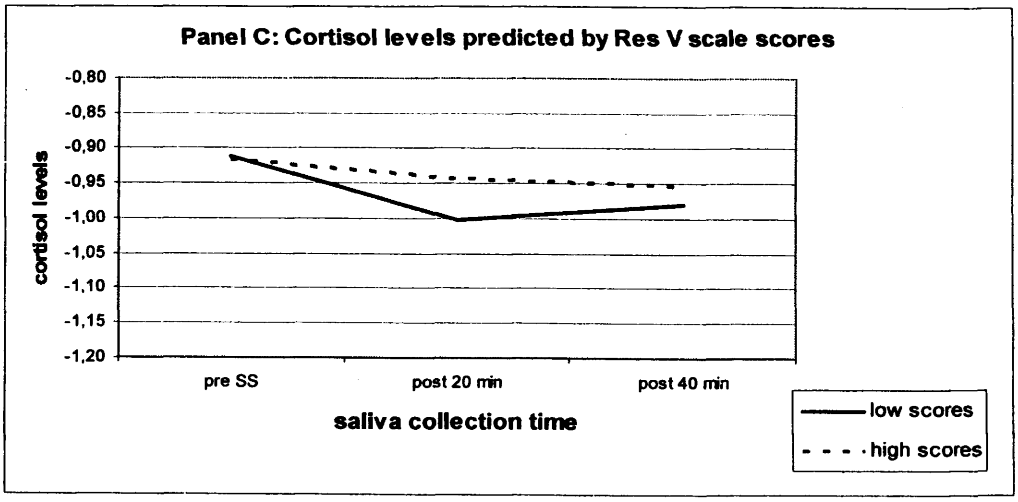
Finally, in order to test which attachment scales, among those found to be associated with cortisol concentrations, had significant independent effects on stress response, "PS VIII","CM VIII", "Re V" "Re VIII", "Av VIII" - jointly with significant child's demographic and state factors - were entered together into the HMLM model. As shown in table 3.9, cortisol levels were found to be predicted by "Resistance to Comforting" scale scores as well as by "Birth order", "Time from awakening to assessment" and "Duration of trip".

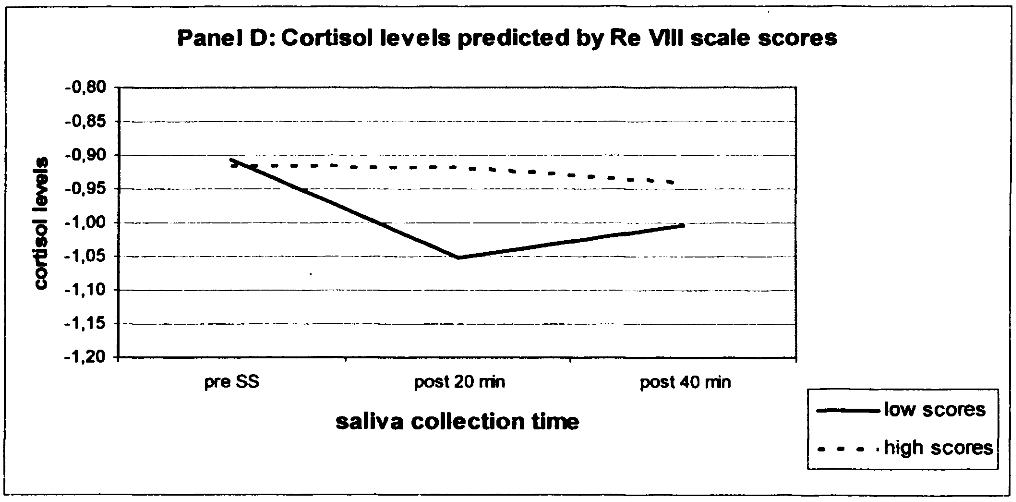
Figure 3.3

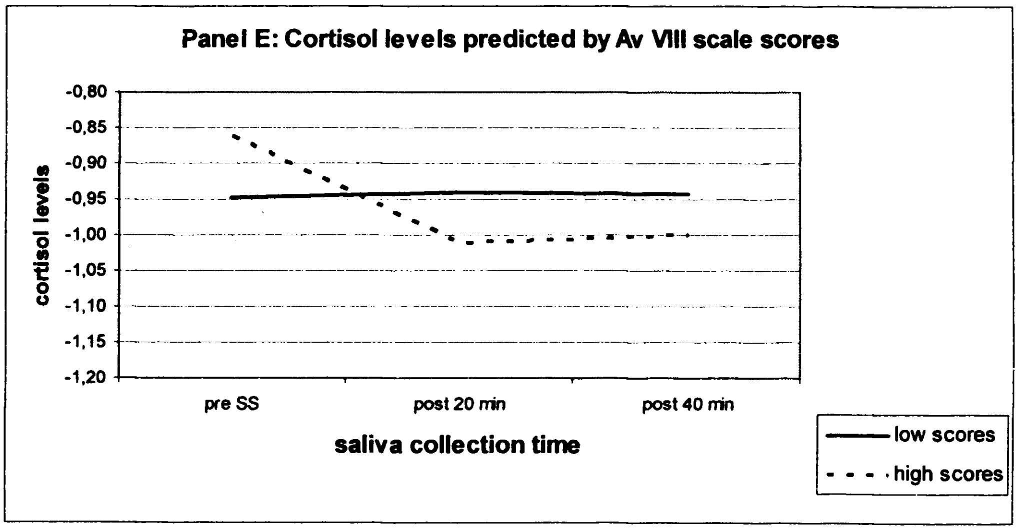
*Cortisol and alpha amylase levels (log-transformed) predicted by PS, CM, Re, Av scales scores*




Panel D: Cortisol levels predicted by Re VIII scale scores
cortisol levels
-0,80
-0,85
-0,90
-0,95
-1,00
-1,05
-1,10
-1,15
-1,20
pre SS
post 20 min
post 40 min
saliva collection time
low scores
high scores


Panel E: Cortisol levels predicted by Av VIII scale scores
cortisol levels
-0,80
-0,85
-0,90
-0,95
-1,00
-1,05
-1,10
-1,15
-1,20
pre SS
post 20 min
post 40 min
saliva collection time
low scores
high scores

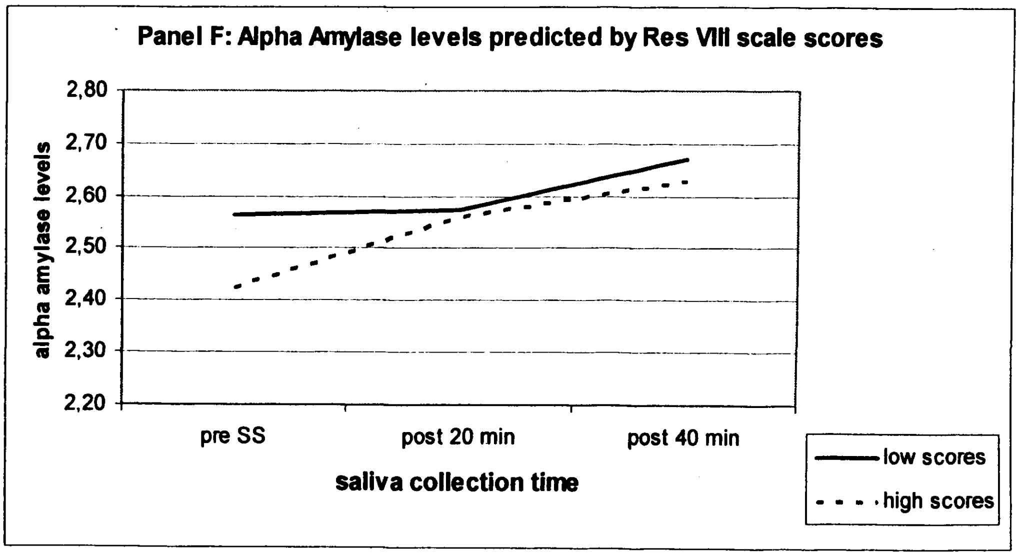

Panel F: Alpha Amylase levels predicted by Res VIII scale scores
alpha amylase levels
2,80
2,70
2,60
2,50
2,40
2,30
2,20
pre SS
post 20 min
post 40 min
saliva collection time
low scores
high scores

Table 3.9

*Attachment scales as predictors of cortisol activity. Final results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | Cortisol | |
| **Proximity Seeking VIII** | 0.60 | 0.75 | -0.70 |
| **Contact Maintaining VIII** | -0.05 | 0.43 | -0.50 |
| **Resistance V** | 0.29 | -0.32 | 0.27 |
| **Resistance VIII** | -0.26 | **2.18*** | **-2.14*** |
| **Avoidance VIII** | 1.37 | -0.42 | 0.19 |
| Birth order | -0.58 | 2.09* | -1.43 |
| Time from awakening to assessment | -2.77** | 3.57*** | -2.99** |
| Duration of trip | 0.23 | 1.63 | -2.40* |

The primary variables of interest for this study (attachment scales) are typed in bold, as well as their significant effect on cortisol data.
* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

## Discussion

The main objective of this study was to investigate adrenocortical and sympatoadrenomedulla activity in infants with child-mother attachment patterns. Although some findings are in line with other investigations, the present study found new data on stress response in infancy which raise intriguing questions in the framework of attachment theory.

Both cortisol and alpha amylase reactivity was not observed in infants classified as secure, thus confirming the role played by attachment security as a social buffer against stress ( Gunnar et al., 1996; Hertsgaard et al., 1995; Nachmias et al., 1996; Schieche and Spangler, 2005; Spangler and Grossman, 1993; Spangler and Schieche, 1998). However, findings related to adrenocortical and sympatoadrenomedulla functioning in insecurely attached infants provide just partial support for the coping model. Specifically, no significant increase in cortisol response was found in infants with insecure attachment but, surprisingly, a significant decrease in cortisol response emerged from pre to post the Strange

Situation in infants classified as avoidant, whereas alpha amylase reactivity was only shown by infants classified as resistant.

Although there is mixed evidence concerning cortisol activation in response to the strange situation in insecure avoidant infants (Hertsgaard et al., 1995; Spangler and Grossman, 1993; Spangler and Schieche, 1998), no previous studies have reported a significant decrease of this hormone in the same lab circumstances. However, some considerations should be kept in mind to better understand this data. First, it is likely that the decline of cortisol concentrations in avoidant infants was related to the high levels of this hormone at the baseline and, consequently, to the recovery afterwards. Alternatively, this data might mirror a potentially disordered functioning of the HPA system, namely "declivity" (see Adam et al., in press). Related to this, a recent study conducted with a low income sample has reported cortisol decreasing in stress response in 6-month-old infants who had low sensitivity mothers (Blair et al., 2006). Specifically, in Blair and colleagues' study (2006) infants of mothers exhibiting lower levels of sensitivity were characterized by higher baseline cortisol and decreasing cortisol levels at 20 and 40 minutes post emotional challenges in comparison with infants of mothers exhibiting higher levels of sensitivity who showed lower baseline and increasing cortisol levels post stressors. Although the age range was slightly different from that of participants of the present study, it is striking how the same pattern of cortisol response emerged in infants who had insensitive mothers and in infants who were avoidant in their attachment relationship in comparison with infants who experienced good maternal care.

Furthermore, previous findings of increased adrenocortical activity in resistant and disorganized infants were not replicated (Hertsgaard et al., 1995; Spangler and Grossman, 1993; Spangler and Schieche, 1998). The lack of adrenocortical activation in resistant infants is in contrast with the coping model which assumes the incapability of these infants to use adequate strategies to regulate their emotional distress, as well as with the distress model which assumes associations between behavioural and physiological activation. The finding of no adrenocortical activation in disorganized infants was also unexpected. A possible explanation might be due to a large proportion (more than 60%) of disorganized infants who had an underlying avoidant attachment pattern. Although the sample size of disorganized infants do not allow comparisons among infants with different underlying attachment organizations (i.e. A, B or C types), avoidant attachment showed a marked cortisol decrease after the stressful procedure which might have masked the effect of disorganization on HPA axis response, as the majority of disorganized infants were also classified as avoidant. In any case the results do not appear to support the interpretation of D as a breakdown of strategy that is evident at the level of psychophysiology, as indicated by these biomarkers.

In respect of the activation of sympatodrenomedulla system in insecurely attached infants, our findings show significant alpha amylase increases after the Strange Situation in resistant infants in comparison with secure infants, but no alpha amylase response in avoidant and disorganized infants. Therefore, in contrast with our findings related to the lack of cortisol response in insecure infants, the activation of sympathoadrenomedulla system in resistant infants is in line with both coping and distress model. In other words, insecure resistant infants showed an activation of the SAM system as they are not able to use a functional strategy to cope with stress (coping model) or, alternatively, as a part of a general stress response along with their behavioural distress (distress model). In contrast, no alpha amylase reactivity was found in avoidant infants thus not replicating data of Hill et al. (2006) who had reported higher alpha amylase levels in avoidant infants in comparison with secure infants. The lack of alpha amylase activation in disorganized infants is in line with data concerning cortisol activation, as well as with other studies (e.g. Spangler and Schieche, 1998) which did not confirm the role played by this attachment pattern in independently predicting physiological stress response.

Temperamental dispositions in stress reactivity and attachment security were then examined in order to test the coping and distress models. Interestingly, behavioural distress significantly predicted cortisol response, whereas attachment status was associated with cortisol baseline levels. Specifically, in line with the distress model infants with low proneness to distress (B1, B2, A) showed a decrease of cortisol levels after the end of the strange situation in comparison with infants with high proneness to distress (B3, B4, C), who showed a slight increase. Consequently, temperamental dispositions are likely to affect adrenocortical reaction to the stress of separation since infants who were more prone to show their distress were also physiologically reactive. On the other hand, we found that insecure infants had higher cortisol levels at the arrival to the lab compared with secure infants, possibly suggesting a significant role played by attachment security in regulating stress response to a new environment. Conversely, no differences in alpha amylase response emerged between infants with low and high proneness to distress as well as between secure and insecure infants. Consequently, while the distress model is not supported by SAM activity in infants who are behaviourally more prone to distress the contribution of the attachment relationship in explaining individual differences in alpha amylase activity may be specifically related to the classification of resistant attachment. However, in order to draw more firm conclusions about the role played by temperament and attachment in shaping physiological stress response a larger sample would be needed to allow comparisons among different groups (A vs B1,B2 vs C vs B3,B4).

Last, individual differences in response to strange situation reunion episodes as measured by scores on attachment scales for assessing ABC patterns were tested in an exploratory fashion in order to better understand the findings from the main analyses of major attachment classifications. In accord with attachment theory, the second separation from the mother represents a more stressful and meaningful situation for infants as separate HMLM analyses revealed that all attachment behaviours significantly predicted HPA axis activity, and resistance to contact behaviours significantly predicted SAM activity in the second reunion. In contrast, no attachment behaviours displayed by infants in the first reunion were found to significantly predict cortisol and alpha amylase reactivity, with the exception of resistant behaviours which were found to be predictive of glucorticoid response.

Thus, cortisol decreases from pre to post the stress of separation were found in infants characterized by a) few efforts to reach proximity to the mother, b) few efforts to maintain contact with the mother, c) few resistant to contact behaviours, and d) a stronger avoidance of the mother. However, further multivariate HLM analyses revealed that resistance to contact during the second reunion was the only independent predictor of adrenocortical reactivity. Specifically, infants with high scores on "resistant to contact" scale had higher post strange situation cortisol levels in comparison with infants with low scores on this scale who showed cortisol decreasing in response to stress. Similarly, the activation of SAM system was more marked in infants who displayed stronger resistance to contact. If this latter finding confirms the role played by resistant attachment in predicting SAM reactivity to stress, the interpretation of findings related to adrenocortical response is less clear and keeps questions on the complex interplay between temperamental and attachment factors open.

In line with previous analyses, the total score on disorganization scale did not predict both cortisol and alpha amylase reactivity, thus suggesting the need of further investigations with larger samples to better understand the role of disorganized attachment in stress response.

To sum up, infants with certain patterns of insecure attachment compared to infants who were securely attached showed an alteration of adrenocortical and sympatoadrenomedulla functioning but the patterns of stress response were distinctive in terms of a deactivation of glucorticoid response in avoidant infants and a hyperactivation of alpha amylase response in resistant infants. Temperamental dispositions to stress were also likely to play a significant role in HPA axis functioning. Although HPA and SAM systems are interconnected physiologically, further evidence is here provided to the fact that they seem to have different characteristic responses to stress and associations with behaviour.

The results of this study should be interpreted in the context of its limitations. First, the small number of participants per attachment groups limits the generalizability of data which require further investigations with larger samples to be replicated. Second, it may be that additional, unmeasured, temperamental traits might contribute to psychophysiological regulation in stressful situations. Furthermore, differences between infants in genetic factors related to HPA activity could add further sources of variation. Therefore, the role played by some child's constitutional factors, such as genes and temperament, in stress response will be explored in the next chapter.

# CHAPTER 4

# THE ROLE PLAYED BY BIOLOGICAL FACTORS IN THE STRESS RESPONSE

## Introduction

The role of biological factors in determining how both animals and humans differ in stress response is documented by several studies (i.e. Bartels et al., 2003; Oswald et al., 2004; Wüst et al., 2004). Specifically, there is broad consensus that genetic and temperamental predispositions contribute to the activity of the Hypothalamic Pituitary Adrenal (HPA) axis and Sympathetic Adreno Medulla (SAM) system in response to challenging circumstances. Most of these studies have focused on salivary cortisol as the main index of HPA axis activity, whereas the role of salivary alpha amylase as a measure of SAM activity (Granger et al., 2007) has not been investigated yet, given that interest in this measure is still recent. Furthermore, although there is some evidence that temperamental characteristics are associated with the stress response as indexed by salivary cortisol and alpha amylase levels in infancy, little is known about genetic influences on the glucorticoid and SAM system responses to stress in this developmental period.

### *Genetic antecedents of stress response*

A relationship between genetic processes and physiological and behavioural reactions to emotional challenges has been suggested many times (Oswald et al., 2004; Smolka et al., 2005; Uhart et al., 2004).

It is estimated that genetic factors account for approximately 50% of interindividual differences in cortisol secretion (Linkowski et al., 1993). Twin studies suggest that genetic factors are likely to be involved in individual differences in stimulated cortisol and ACTH responses (Froelich et al., 2000); furthermore, some mental disorders, such as panic disorders, which affect the sympathetic system and hypothalamic-pituitary-adrenal axis functioning have moderate to high (30%-40%)

inherited vulnerability (Kendler et al., 1993). In addition to twin studies, molecular genetic studies can help to better understand the contribution of specific candidate genes in determining individual differences in response to stressful circumstances.

Since norepinephrine (NE), dopamine and serotonin play important roles in regulating the stress response, it is reasonable to hypothesize that genetically determined differences in the functioning of these neurotransmitters might influence HPA axis and SAM activity. Plausible candidate genes involve coding and regulating DNA that influence the transcription and functional activity of neurotransmitter receptors and the enzymes involved in neurotransmitter metabolism (e.g. Jabbi et al., 2007).

Catechol-Omethyltransferase (COMT) is an enzyme involved in the breakdown of the catecholamine neurotransmitters, dopamine, epinephrine and norepinephrine. Interestingly, a common functional polymorphism in the COMT gene has recently been associated with psychiatric conditions, such as bipolar disorders (Papolos et al., 1998), obsessive compulsive disorder (Karayiorgou et al., 1997), and alcoholism (Tiihonen et al., 1999; Wang et al., 2001), as well as with differences in prefrontal cognitive perfomance (Malhotra et al., 2002). This polymorphism of the COMT gene is observed at relatively high rates in the population and involves a substitution of valine (val) by methionine (met) at codon 158 (val158-met). The polymorphism is associated with a difference in thermo stability leading to a three–four fold reduction in the activity of the COMT enzyme in catabolizing dopamine, epinephrine (EP), and norepinephrine (NE) (Lotta et al., 1995; Lachman et al., 1996). The alleles are codominant so that individuals with the val/val genotype have the highest activity of COMT, those with the met/met genotype have the lowest activity of COMT, and heterozygous individuals are intermediate (Scanlon et al., 1979).

The met allelic loading has been identified as a marker of excercebated endocrine response to stress. Recently, the COMT met allele has been associated with anxiety and increased cortical response to negative emotions (Enoch et al., 2003; Smolka et al., 2005). Furthermore, Oswald and colleagues (2004) found that adrenocorticotropin hormone and cortisol responses to naloxone challenge were greater in individuals with met genotypes compared to individuals homozygous or heterozygous for the valine allele. Last, a recent investigation from a Dutch group

(Jabbi et al., 2007) has found that individuals homozygous on the met allele had higher endocrine and reported subjective stress responses. However, no studies have investigated the role of the COMT polymorphism to stress responses in children or infants.

Although several different genes are known to contribute to the regulation of serotonergic and dopaminergic functions, the Serotonin transporter (5-HTT) and the Dopamine D4 receptor (DRD4) gene have been widely explored -alone or in combination- as possible contributors to variation in both normal traits and psychopathology, such as anxiety and depression, in adults and children (Ebstein et al., 2000; Oak et al., 2000; Reif and Lesch, 2003).

The serotonin transporter (5-HTT) has received particular attention because it is involved in the re-uptake of serotonin at brain synapses and is the target of SSRIs (Selective Serotonin Reuptake Inhibitors). The activity of the 5-HTT gene is affected by a polymorphism in the proximal 5' promoter region, designated the 5HTT gene-linked polymorphic region (5-HTTLPR). The short (S) allele in the 5-HTTLPR is associated with lower transcriptional efficiency of the promoter compared with the long (L) allele (Lesch et al., 1996). Both adults and children with the L-L genotype have significantly higher serotonin uptake compared to those with L-S or S-S genotypes (Greenberg et al., 1999; Nobile et al., 1999). This genetically-influenced functional difference in serotonin uptake appears to influence a significant proportion of phenotypic variation in several human behaviors, including negative emotionality, interpersonal hostility, aggressiveness, and proneness to anxiety/depression (see e.g., Battaglia et al., 2005; Nobile et al., 2004, and Lesch, 2002 and Munafò et al., 2003 for reviews). More interestingly, serotonin transporter gene disruption in rodents produces anxious animals with exaggerated limbic-hypothalamic pituitary adrenal responses to stress (Lanfumey et al., 2000; Li et al., 1999; Wichems et al., 2000), and a recent study by Caspi et al. (2003) in humans found the S allele to be associated with depression but only among individuals exposed to major life stressors.

The DRD4 is a G-protein coupled receptor belonging to the D2 receptor family which exerts an inhibitory effect on the adenylate cyclase-mediated secondary messenger pathway (Kandel, 2000). The DRD4 gene is situated on the long arm of chromosome 11 (Gelertner et al., 1992; Petronis et al., 1993), with a 48 base-pair VNTR polymorphism in the third exon constituting the main focus of attention.

Between 2 and 11 repeated elements have been reported in the literature, although the two predominant alleles in Caucasians consist of four (4R) and seven (7R) repeats (Ding et al., 2002; Lichter et al., 1993; Van Tol et al., 1992). The 7-repeat allele has been found to have a lower potency for dopamine-mediated coupling to adenylate cyclase than receptors encoded by the 2-or 4- repeat forms (Asghari et al., 1995). Furthermore, new results suggest that the 7-repeat variant of the gene is significantly less efficient at the levels of transcription, translation and second messenger generation compared to the most frequent short forms (Schoots and Van Tol, 2003; Van Craenenbroeck et al., 2005). Evidence is accumulating that links the 7-repeated allele of the DRD4 gene with infant temperament traits of negative emotionality (Auerbach et al., 1999; De Luca et al., 2001), maladaptive behavioral problems, especially attention deficit/hyperactivity disorder (ADHD) in children (e.g. Faraone et al., 2005; LaHoste et al., 1996; Swanson et al., 2000). Furthermore, the 7-repeat DRD4 allele was found to be 2.5 times more frequent among one-old years infants with a pattern of disorganized attachment to their mothers (Lakatos et al., 2000), which is thought to indicate an inability to cope with stress (Hesse and Main, 2000). Additionally, the association between the 7-repeat allele and disorganized attachment was shown to be enhanced in the presence of another polymorphism of the same gene (Lakatos et al., 2002): the –521 C/T single nucleotide polymorphism (SNP), which is a C>T substitution in the promoter region of the DRD4 gene. It has been found that in a human retinoblastoma cell line, the –521 T allele reduced transcriptional efficiency by approximately 40% compared to the –521 C variant (Okuyama et al., 1999), thus suggesting the functional relevance of this polymorphism for dopaminergic neurotransmission. Lakatos and colleagues (2002) found that in the presence of both risk alleles (7 repeat allele and –521 T allele) the odds ratio for disorganized attachment increased tenfold. Moreover, the -521 C/T SNP was shown to be associated with temperament traits, namely novelty seeking (Okuyama et al., 2000; Ronai et al., 2001). The CC variant has also been found to be more frequent in schizophrenic patients (Okuyama et al., 1999).

To sum up, some empirical evidence directly supports the role of COMT and 5-HTT polymorphisms in physiological reactions to stress, while the involvement of DRD4 and DRD4/521 polymorphisms, though quite plausible has not been directly tested. Furthermore, most existing studies of genetic effects have looked at cortisol, and none have investigated the possible genetic contributions to alpha amylase response.

*Temperament and stress response*

Differences in temperament and personality influence how individuals experience potentially challenging stimuli (Kendler et al., 2003; Puttonen et al., 2005). Behavioural inhibition is the trait that has been most strongly linked to HPA axis hyperactivity, as it has been extensively studied in human children and adults and has apparent analogues in rodents and non-human primates. Humans and animals with this trait exhibit a stable behavioural tendency to avoid or withdraw from novel situations (Fox et al., 2005; Kalin and Shelton, 2003). Behavioural inhibition might influence how a child evaluates a stressor as well as the coping strategies the child chooses; another hypothesis is that it might be related to a lower activation threshold in the limbic areas of the brain, especially the amygdala. One of the stress-sensitive systems in which the amygdala is involved, at least in part, is the HPA system, the end product of which in humans is cortisol (Kagan et al., 1987, 1988; Gunnar, 1994; Gunnar et al., 1997).

Research on children's temperamental characteristics and activity of the HPA system provides mixed evidence for how these constructs are related. A longitudinal study (Kagan et al., 1987) of two cohorts of 2 and 3 year-old children, assessed as extremely cautious and inhibited or fearless and uninhibited to unfamiliar events, revealed that those who were extremely inhibited showed at 5 years higher levels of salivary cortisol both at home and during a series of unfamiliar and challenging events in the laboratory than did extremely fearless and outgoing children. Similarly, Schmidt and colleagues (1997) showed that 4 year old children who were extremely wary of social novelty during peer play exhibited relatively higher morning salivary cortisol compared with other children. Furthermore, Flinn and England (1997) found a positive association between shyness and high cortisol values in a sample of Caribbean children. However, although several studies have generally found that a higher level of the stress hormone cortisol is associated with shy, withdrawn behaviour, work on salivary cortisol has also indicated that a higher level of cortisol within a moderate range is positively associated with approach behaviour, social competence, and cognitive and behavioural inhibitory control in children and adults

(Blair et al., 2003; Davis et al., 2002; Erickson et al., 2003). Moreover, other studies, particularly in the study of preschool children in day care settings, have indicated that children characterized by higher levels of approach, also referred to as surgent, extroverted children, tend to exhibit higher levels of cortisol than do shy, withdrawn children (Gunnar et al., 1997). Finally, a study aimed also to evaluate the relationship between temperament and physiologic response to immunization in infancy found that cortisol responses were related to the temperament characteristics of intensity, rhythmicity, approach, withdrawal, and distractibility in infancy (Wilson et al., 2003).

The study of temperament and personality has indicated a role for autonomic nervous system (ANS) function in emotional and stress-related reactivity associated with response to appetitive and aversive motivational stimuli (Derryberry and Reed, 1994; Gray, 1972; Rothbart and Derryberry, 1981). Low autonomic arousal, primarily indicated by low resting heart rate and perhaps indicative of sympathetic underarousal, has been associated with aggression and antisocial behaviour in both children and adults (Raine, 2002). In contrast, some studies have shown heightened sympathetic reactivity in inhibited children, thus providing further physiological support for a relationship between inhibition and limbic activation (Kagan et al., 1987; Scarpa et al., 1997). For example, Scarpa and colleagues (1997) found that inhibited 3 year-old children displayed higher heart rate and skin conductance levels and longer skin conductance latency compared to uninhibited children during a tone task.

However, studies aimed at exploring the association between temperament characteristics and SAM response to stress as indexed by alpha amylase levels are still very scarce. An extensive review by Granger et al. (2007) on the role of salivary alpha amylase in biobehavioural research refers to just one study about this issue. Specifically, El Sheikh and colleagues (*under review*) examined relations between salivary cortisol, alpha amylase collected before and after two laboratory stressors, and problem behaviours in a sample of 8-9 years old children. The authors found that post-stress salivary alpha amylase was predictive of girls' internalizing behaviours (e.g., social withdrawal, anxiety/depression); furthermore, higher salivary alpha amylase reactivity from pre to post lab stressors was associated with higher levels of internalizing symptoms in girls. Although this is the first study which has looked into salivary alpha amylase response in relation to some behavioural problems in childhood such as social withdrawal, likely to characterize inhibited children, further studies more focused on the investigation of specific temperament characteristics are needed in order to better understand the relation between temperament and alpha amylase levels in response to stress.

This study aims to address the extent to which candidate genetic polymorphisms and gene X gene (DRD4 X DRD4/521) interactions contribute to infant's physiological responses to stress in the HPA and SAM systems during a stressful procedure (Ainsworth's Strange Situation). The study also tests the role played by temperamental traits in cortisol and alpha amylase responses.

## Methods

*Participants*

The initial sample was composed of 82 health infants (45 boys) aged 12 to 18 months (mean age = 14.6; SD = 1.8) and their mothers (mean age = 34.2; SD = 4.3) belonging to middle class families. The final sample included 72 and 74 infants with cortisol and alpha amylase data respectively as well as temperament data, as 10 (9.7%) and 6 (7.3%) infants were excluded from the analyses because of the lack of any cortisol (N = 8) and alpha amylase (N = 6) data and 2 infants were excluded because mothers did not completed the Toddler Temperament Scale (Fullard, McDevitt, Carey, 1984). Genotyping of DRD4, DRD4/521, 5-HTTPLPR and COMT was not respectively successful for 5, 2, 4 and 1 infants. Signed informed consent was obtained from the mothers for participating in the whole study.

The assessment procedure took place in the Psychopathology Unit of the "Eugenio Medea" Scientific Institute, where the assay of cortisol and the genotyping were also been performed. Saliva was collected before the Strange Situation as well as 20 and 40 minutes after the procedure. Mothers also completed a brief socio-demographic form and provided information regarding those factors which can affect basal cortisol and alpha amylase levels (see methods section of chapter 2 for a full description of both forms). The non-invasive sampling of buccal cells by cotton swabs, and the Toddler Temperament Scale by parent were collected at the end of the assessment session.

The research protocol has been approved by the Ethical Committee of University College London and "Eugenio Medea" Scientific Institute.

*Instruments*

**Stressor.** The Strange Situation Procedure (Ainsworth et al., 1978) was used as stressor. This procedure involves a structured series of eight episodes in which infant is gradually exposed to mildly stressful events. Specifically, infants have to cope with an unfamiliar setting where the entrance of an unfamiliar adult and two separations from the parent, followed by a reunion are prearranged. A full description of the Strange Situation procedure and its evaluation is reported in the methods section of chapter 3.

**The Toddler Temperament Scale.** The Toddler Temperament Scale (Fullard, McDevitt, Carey, 1984) is a questionnaire filled out by parents whose aim is to evaluate the temperament of infants aged 1 to 2 years olds. It is composed by 92 items which are rated on a 6 point scale (from 1 = hardly ever to 6 = almost always). It evaluates toddler temperament according to the 9 dimensions identified by Thomas and Chess (1977): 1) Activity (level and extension of motor activity); 2) Rhythmicity (regularity of biological functions, as sleep and feeding); 3) Approach (first reaction of the toddler to new stimuli); 4) Adaptability (facility of adjustment to changes); 5) Intensity (or energetic level of the responses; i.e."Shows strong reactions towards failures"); 6) Mood (quantity of the behaviours which express a positive state of mind opposed to the quantity of distress and discouragement manifestations); 7) Persistence (or amount of time spent on the same activity); 8) Distractibility (effect of the external stimuli on the present activity) and 9) Threshold (level of stimulation requested for having a reaction). Mean scores at each scale, ranging from one to six, identification of infants who are "very easy" (mean scores close to 1) and "very difficult" (mean scores close to 6) from the above behavioural dimensions.

The Italian version of the Toddler Temperament Scale has been translated and validated (Axia, 1993) and shows good psychometric properties.

*Psychophysiological assessment*

**Salivary Cortisol /Alpha Amylase Collection and Assay.** Saliva samples were collected by asking the child to suck/chew on a sterile cotton dental roll - lightly dusted on the tip with 2 or three grains of Kool Aid crystals - until the cotton was saturated enough to fill a small vial. Alternatively, if the child refused to chew on the cotton roll a microsponge (Sorbette -Salimetrics) was placed in the child's mouth for around 1 minute to collect saliva. Saliva samples were stored at – 80° C until the assay. Saliva was extracted from sorbettes by centrifuging the cryovials (where the sorbettes had been inserted in) for 20 minutes at 1500 Xg before the assay.

The assessment of cortisol levels was done by two competitive immunoassays specifically designed for the quantitative measurement of salivary cortisol (HS-Cortisol EIA Kit and Expanded Range (ER) HS-Cortisol EIA Kit, Salimetrics) in the biology lab of the "Eugenio Medea" Scientific Institute. To guarantee validity of analysis all samples from one infant were analyzed in one assay, and duplicate assays were performed whenever possible. Average intra-and interassay coefficients of variation were less than 9% and 10% respectively. Cortisol levels were not affected by saliva collection methods (rope versus sorbette), cortisol assay (duplicate versus singlet) and cortisol kits (see chapter 2).

The assessment of alpha amylase levels was done by a commercially available kinetic reaction assay (Salimetrics, State College, PA) in the Salimetrics lab (University of Pennsylvania). The test has a reported average inter assay variation computed for the mean of average duplicates for 8 separate runs for lower (10,6 U/mL) and higher (166.0 U/mL) concentrations samples were 5,8 and 3,6 % respectively whereas intrassay variation computed for the mean of 10 replicate tests of low (17,7% U/mL), medium (108,8 U/mL), and high (474,6 U/mL) concentrations samples were 7,2%, 6,7%, and 2,5%, respectively (Granger et al, In Press). Alpha amylase levels were not affected by saliva collection methods (rope versus sorbette) (see chapter 2).

A full description of saliva cortisol/alpha amylase collection and assay is reported in chapter 2.

*Genetic Assessment*

**Buccal Sample collection and DNA extraction.** Buccal epithelial cells were collected by swabbing the inside of the infant's mouth for approximately 20 seconds using a regular cotton wool bud (e.g. Q-swab, one of the cotton-ends cut). The procedure was repeated with a second swab on a different area of the mouth. The swabs were stored at +4°C before processing. Each swab was processed by soaking it in 1 ml of buffer containing 10 mM NaCl, 10 mM Tris HCl pH8, 10 mM EDTA pH 8 in a 1.5 ml Eppendorf tube, vortexed for 20'', then the swab was taken out and the sample was centrifuged for 1' at 12,000 rpm in a microfuge. The supernatant was discarded and the pellet frozen pending DNA extraction.

Genomic DNA was extracted with DNAzol Genomic DNA isolation reagent (MRC, Cincinnati, OH). In detail, each pellet was resuspended in 0.5 ml of DNAzol supplemented with 5 μl of Polyacryl Carrier (MRC) and stored for 10 min at room temperature (RT). The homogenate was sedimented for 10 min at 10,000 g at RT, and the supernatant was transferred to a new 1.5 ml tube. DNA was precipitated by addition of 250 μl of 100% ethanol and stored at RT for 3 min, then sedimented by centrifugation at 5,000 g for 5 min at RT. The precipitate was washed twice with 1 ml of 75% ethanol, drained thoroughly and dissolved in 150 μl of 8 mM NaOH. When the pellet had fully dissolved, pH was adjusted to 7.5 by the addition of 24 μl of 100 mM HEPES and the DNA was stored at +4°C. One μl of DNA was used for each amplification reaction.

**Genotyping.** The DRD4 gene is located on 11p15 between HRAS and tyrosine hydroxylase genes. A highly interesting polymorphism has been discovered consisting in a set of variants in the portion of the DRD4 gene coding for the third transmembrane (TM3) region of the receptor. The variations occur as the result of the insertion of a variable number of copies of a 48-bp sequence located in the exon coding for the third cytoplasmic loop. Most important is the fact that different variants of the gene code for receptor proteins that exhibit significant differences in pharmacologic affinity for spiperone and clozapine.

PCR was carried out with the following primers: GCG ACT ACG TGG TCT ACT CG (on the 5' side of the polymorphism for the third cytoplasmic loop) and AGG ACC CTC ATG GCC TTG (on the 3' side of the polymorphism).

Reactions were carried out in 25 μl of a mix containing 1x Taq Gold polymerase buffer, 1.5 mM $Mg^{++}$, 0.2 mM dATP, dCTP, dTTP, 0.1 mM dGTP, 7-deaza-dGTP, 10% DMSO, 0.5 μM of each primer, 1 U Taq Gold (Perkin-Elmer Co., Norwalk, CT), and 0.1 μg genomic DNA. The amplification profile was: 9 min at 95°C, 35 cycles of 15 s at 94°C, 15 s at 54°C, 20 s at 72°C, 72°C chase for 4 min. Amplification was performed on a GenAmp 2400 PCR system (Perkin-Elmer).

Amplification products were analysed on a 4% (3:1 NuSieve GTG / Standard) agarose / TAE gel. The alleles were numbered according to the number of 48-bp repeats contained in each fragment.

A 372 bp *DRD4* promoter fragment carrying the –521 position was amplified using the primers F (5'-GGC GGC CAC GCG AGG ATC AAC TGT GC-3') and R (5'-CGG CCA GAC CAG GCC CTG AAG C-3'). The fragment was amplified by an initial denaturation step of 5 min at 95°C, followed by 38 cycles of 45 sec at 95°C, 30 sec at 60°C, 1 min at 72°C, and a final extension step of 5 min at 72°C. The PCR fragments were sequenced using primer R and dye terminator chemistry (BigDye v3.1, Applied Biosystems). Sequencing reactions were run and analyzed on an ABI PRISM 3130xl capillary sequencer.

The polymorphism in the transcriptional control region upstream of the 5-HTT gene coding sequence (5-HTTLPR) was analyzed by polymerase chain reaction (PCR) according to the method reported by Lesch et al. (1996). Two fragments were generated: a short variant (*s*) of 484-bp, and a long variant (*l*) of 528-bp.

All amplification reactions were performed on a Gene Amp PCR System 9600 Thermocycler (Perkin-Elmer). The amplified products were analyzed on 4% (3:1 NuSieve GTG: Standard) agarose gels.

The *COMT* gene contains a G to A missense variant (Lachman et al., 1996) that translates into a substitution of methionine for valine at codon 158 (*val158met*), with the enzyme containing *met158* having one third to one-fourth of the activity of the *val158* enzyme (Lotta et al., 1995) in degrading dopamine, epinephrine,and norepinephrine. We determined the subject's *COMT* V158M genotypes using the 5'-exonuclease Taqman assay (Chen et al., 2004). The Taqman primers, probes and reagents were purchased from Applied Biosystems. The assays were performed and analyzed on a 9700HT Sequence Detection System (Applied Biosystems).

All the genotyping were done in the genetic laboratory of the Eugenio Medea Scientific Institute, blind to all the other variables investigated in the study (ie. attachment status and psychophysiological outcomes).

*Grouping*

DRD4 genotypes were grouped according to absence (7-) vs. presence (7+) for the 7 - repeat allele in line with Lakatos et al. studies (2000; 2002;2003) so that subjects could be split into DRD4 7- vs. DRD4 7+ (encompassing homozygosity and heterozygosity for 7 allele) carriers. In the present sample, this grouping is almost the same of the short (2-5 repeats) vs long (6-8 repeats) scheme (Benjamin et al., 1996; Ebstein et al., 1996) for grouping DRD4 genotypes (there was only one infant with the genotype 4/9) which is often used (Asghari et al. 1994; 1995) but not recommended by other authors (Wong et al., 2000) who call attention to the non linear nature of binding properties of alleles of different length. For the DRD4/521 polymorphism, participants were classified into C and T (encompassing T and C/T) allele carriers while for the COMT genotype participants were classified into AA and G (encompassing G and G/A) allele carriers. Last, for the 5-HTTLPR genotype the sample was split into S-allele carriers and LL subjects (encompassing LL and SL) while one SI allele carrier infant was excluded from the analyses.

## Results

In the following paragraphs, the effects of genetics and temperament on salivary cortisol and alpha amylase (A-A) activity will be tested by hierarchical multivariate linear model (HMLM) analyses. Preliminarily, the distribution of genotype and allele frequencies and percentages in the sample will be shown and the Hardy Weinberg equilibrium for each polymorphism will be tested; moreover, the distribution of the 521 C/T genotypes between groups with and without the 7-repeat allele will be investigated to exclude a possible association between these polymorphisms of the DRD4, whose interaction will be subsequently tested. Last, chi square analyses will be run to control for possible associations between polymorphisms and socio-demographic variables. Similarly, preliminary analyses will be performed to check for the effect of potential interfering variables on temperament scales. Specifically, separate ANOVAs will be run to analyze whether sociodemographic factors have an effect on temperamental traits.

Findings about genes and temperament will be reported separately.

*Genetics and physiology: preliminary analyses.*

Table 4.1 shows the genotype and allele frequencies and percentages in the sample for each polymorphism. For the DRD4, in agreement with data for other mixed European and Caucasian populations (Chang et al., 1996; Strobel et al., 1999) the 4 repeat allele was represented with the highest frequency, followed by the 7 repeat allele. Similarly, DRD4-521, 5-HTT and COMT allele frequencies were similar to those in populations of European descent (e.g. Lakatos et al., 2002; 2003; Oswald et al., 2004).

All genotypes were in Hardy-Weinberg equilibrium (5-HTT: $\chi^2$ (1) = 1,61, $p > 0.20$; COMT: $\chi^2(1) = 0.24$, $p > 0.50$; DRD4: $\chi^2$ (1) = 0.001, $p$ = ns) with the exception of DRD4/521 ($\chi^2$ (1) = 7.82; $p < 0.01$ ).

The distribution of the -521 C/T genotypes did not differ significantly between groups with and without the 7-repeat allele ($\chi^2$ (1) = 0.48 $p = 0.34$).

In order to test eventual associations between each candidate polymorphism and sociodemographic factors, chi-square analyses were performed. No significant associations were found.

Table 4.1
*Genotype and allele frequencies and percentages*

| *Genotype* | f | % | ***allele*** | | | | | |
|---|---|---|---|---|---|---|---|---|
| *COMT* | | | **G** | **A** | | | | |
| G | 24 | 29.6 | 48 | - | | | | |
| AA | 16 | 19.8 | - | 32 | | | | |
| GA | 41 | 50.6 | 41 | 41 | | | | |
| *Total* | 81 | 100.0 | | | | | | |
| *5- HTT* | | | **L** | **S** | | | | |
| LL | 30 | 38 | 60 | - | | | | |
| SS | 16 | 20.4 | - | 32 | | | | |
| SL | 32 | 40.5 | 32 | 32 | | | | |
| SI | 1 | 1.3 | | | | | | |
| *Total* | 78 | | | | | | | |
| *DRD4-521* | | | **C** | **T** | | | | |
| CC | 24 | 30 | 48 | - | | | | |
| TT | 28 | 35 | - | 56 | | | | |
| CT | 28 | 35 | 28 | 28 | | | | |
| *Total* | 80 | | | | | | | |
| | | | **2** | **3** | **4** | **7** | **9** | |
| DRD4 | | | *12* | *5* | *112* | *24* | *1* | **N =** |
| | | | *(7.8)* | *(3.2)* | *(72.7)* | *(15.6)* | *(0.6)* | **154** |
| 2/2 | 1 | 1,3 | 2 | | | | | |
| 2/3 | 2 | 2,6 | 2 | 2 | | | | |
| 2/4 | 6 | 7,8 | 6 | | 6 | | | |
| 2/7 | 2 | 2,6 | 2 | | | 2 | | |
| ¾ | 2 | 2,6 | | 2 | 2 | | | |
| 3/7 | 1 | 1,3 | | 1 | | 1 | | |
| 4/4 | 43 | 55,8 | | | 86 | | | |
| 4/7 | 17 | 22,1 | | | 17 | 17 | | |
| 4/9 | 1 | 1,3 | | | 1 | | 1 | |
| 7/7 | 2 | 2,6 | | | | 4 | | |
| *Total* | 77 | | | | | | | |

Genetics and physiology

In order to test if each genetic polymorphism predicted cortisol and A-A levels response to stress, separate HMLM analyses were conducted. For cortisol, “birth order”, “time from awakening to assessment”, and “duration of trip” - which were found to significantly predict cortisol data (see chapter 2) - were entered into the model jointly with each genetic polymorphism; similarly, for alpha amylase, “time from breakfast to assessment” and “medication” - which were found to significantly predict A-A data (see chapter 2) - were entered into the model together with genetic polymorphisms. As shown in table 4.2, no genotype predicted cortisol or A-A concentrations with the exception of DRD4/521 genotype which predicted baseline A-A levels.

Table 4.2 *Genetic polymorphisms as predictors of cortisol and A-A activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | *Cortisol* | |
| **5-HTT SS** | 0.55 | -1.53 | 1.37 |
| **5-HTT SL** | -0.17 | 0.17 | -0.37 |
| Birth order | -0.74 | 1.85 | -1.20 |
| Time from awakening to assessment | -2.76** | 3.53*** | -2.12* |
| Duration of trip | 0.84 | 1.29 | -2.22* |
| **COMT AA** | -1.32 | 0.54 | -0.11 |
| **COMT AG** | 0.29 | -0.52 | 0.27 |
| Birth order | -0.80 | 2.27* | -1.54 |
| Time from awakening to assessment | -2.72** | 3.61*** | -3.12** |
| Duration of trip | 0.57 | 1.03 | -1.85 |
| ***DRD4 (7+ vs. 7-)*** | -0.51 | 0.71 | -1.13 |
| Birth order | -1.03 | 1.74 | -0.95 |
| Time from awakening to assessment | -3.23** | 5.55*** | -5.36*** |
| Duration of trip | -0.33 | 1.59 | -2.35* |
| **DRD4/521 TT** | -0.13 | -1.71 | 1.72 |
| **DRD4/521 CT** | 1.27 | -0.88 | 0.41 |
| Birth order | -1.26 | 2.57** | -1.84 |
| Time from awakening to assessment | -3.09** | 3.52*** | -3.05** |
| Duration of trip | 0.66 | 1.45 | -2.35* |
| | | *Alpha Amylase* | |
| **5-HTT SS** | 0.67 | 0.62 | -0.81 |
| **5-HTT SL** | 0.24 | -1.15 | 1.49 |
| Time from breakfast to assessment | 0.43 | -2.27* | 2.04* |
| Medication | 0.66 | -2.28** | 2.27* |
| **COMT AA** | -1.10 | 0.33 | -0.53 |
| **COMT AG** | -1.01 | 0.44 | -0.30 |
| Time from breakfast to assessment | 0.71 | -2.57** | 2.37* |
| Medication | 0.68 | -1.92 | 1.83 |
| **DRD4 (7+ vs. 7-)** | 0.33 | 0.97 | -1.28 |
| Time from breakfast to assessment | 0.14 | -2.07* | 1.69 |
| Medication | 0.61 | -2.36* | 2.13* |
| **DRD4/521 TT** | -1.63 | -0.35 | 0.78 |
| **DRD4/521 CT** | **-2.47**** | 0.70 | -0.52 |
| Time from breakfast to assessment | 0.02 | -2.16* | 1.99* |
| Medication | 0.86 | -2.03* | 1.92 |

The primary variables of interest for this study (genes) are typed in bold, as well as their significant effect on cortisol and alpha amylase data.
* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

Specifically, DRD4/521 CC allele carriers had significantly higher A-A levels than DRD4/521 CT allele carriers as shown in Fig. 4.1.

HLML analyses were also run with 5-HTT, DRD4/521, and COMT genotypes grouped into "at risk" and "not at risk" allele carriers (see methods section), taking also into account the effects of predictive demographic and child's state factors. None of these polymorphisms significantly predicted salivary cortisol and alpha amylase values with the exception, again, of DRD4/521 on alpha amylase levels (intercept: $T = -2.29$; $p = 0.02$). However, a modest trend of 5-HTT (SS vs LL and LS) genotype to predict cortisol levels was found as shown in table 4.3. Specifically, SS allele carriers infants tended to show a decrease of cortisol levels after the stressor in comparison with LL and SL carriers infants who showed more stable levels across the three saliva collections (Fig. 4.2).

Last, DRD4 X DRD4/521 interaction was tested as potential predictor of cortisol and alpha amylase levels, since theoretical and empirical relevance of its influence has been provided (Lakatos et al., 2002; 2003). However, no significant effect of this gene X gene interaction on physiological measures was found (table 4.4).

Figure 4.1
*Alpha Amylase levels (in U/mL) predicted by DRD4/521 genotype. Results of the HMLM analyses*

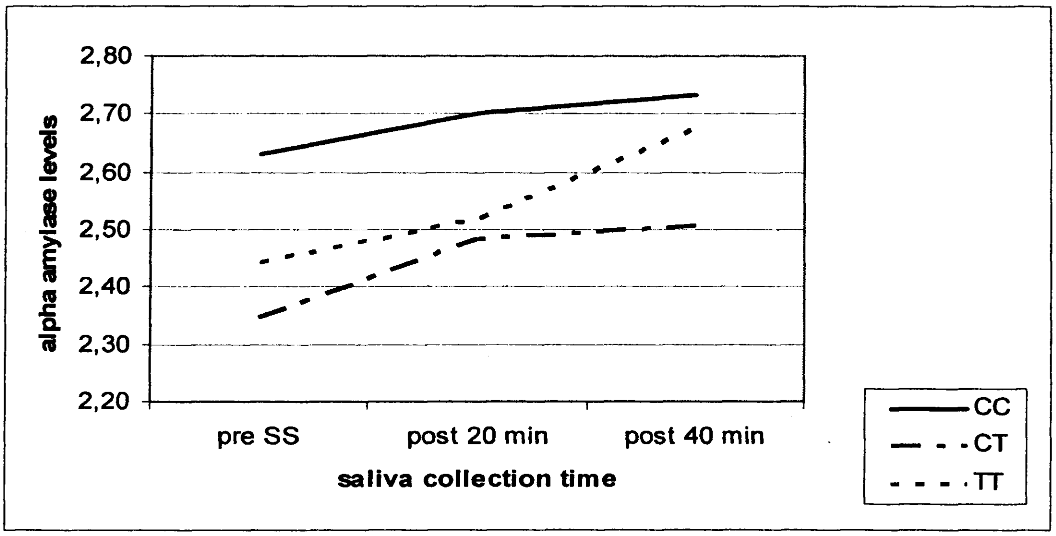



Figure 4.2

*Cortisol reactivity (in μg/dl) predicted by 5-HTT genotype. Results of the HLML analyses*

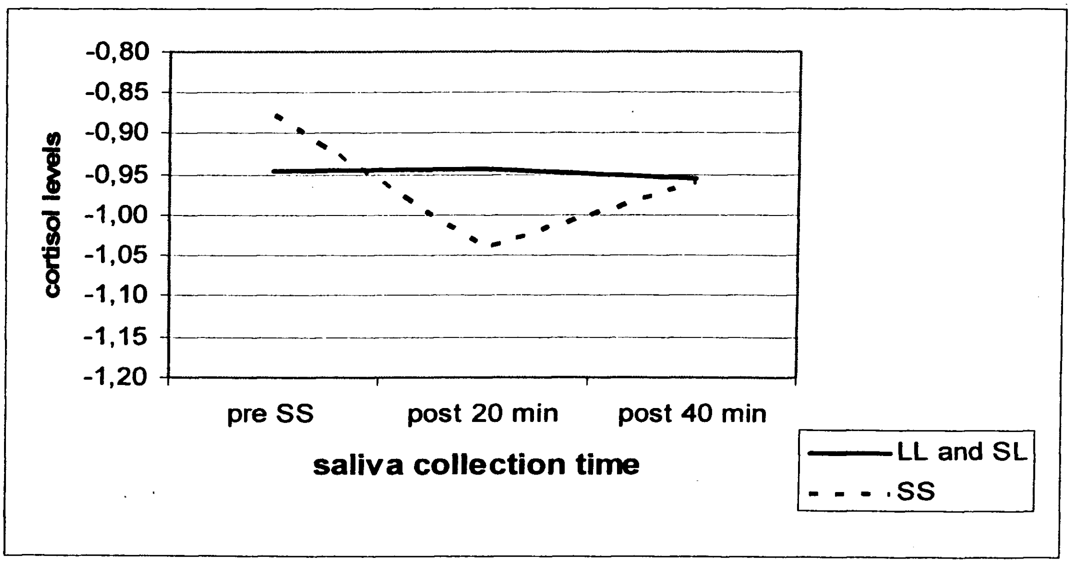


Table 4.3

*5-HTT genotype as predictor of cortisol and A-A activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | *Cortisol* | |
| **5-HTT** | 0.70 | -1.82[t] | 1.77 |
| Birth order | -0.78 | 1.85 | -1.21 |
| Time from awakening to assessment | -2.77** | 3.48*** | -2.92*** |
| Duration of trip | 0.82 | 1.29 | -2.20* |

The primary variable of interest for this study (5-HTT) is typed in bold, as well as its significant effect on alpha amylase data.
p<0.05; ** p < 0.01; *** p < 0.001; [t] p = 0.069

Table 4.4

*Interactions between polymorphisms in predicting cortisol and alpha amylase activity. Results of the HMLM analyses*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | *Cortisol* | |
| **DRD4 x DRD4/521** | 1.17 | -0.08 | 0.30 |
| **DRD4** | -1.07 | 0.32 | -0.76 |
| **DRD4/521** | 0.32 | -0.99 | 0.61 |
| Birth order | -1.87 | 2.08* | -1.09 |
| Time from awakening to assessment | -3.35** | 5.59*** | -5.49*** |
| Duration of trip | -0.44 | 1.63 | -2.46* |
| | | *Alpha Amylase* | |
| **DRD4 x DRD4/521** | -1.64 | 1.52 | -1.55 |
| **DRD4** | 1.46 | -0.63 | 0.49 |
| **DRD4/521** | -0.89 | -0.69 | 0.89 |
| Time from breakfast to assessment | -0.31 | -2.00* | 1.67 |
| Medication | 0.52 | -2.38* | 2.18* |

The primary variables of interest for this study (genes) are typed in bold, as well as their significant effect on cortisol and alpha amylase data.
* p<0.05; ** p < 0.01; *** p < 0.001

*Temperament and physiology*

Preliminary analyses

In order to test if sociodemographic factors had an effect on temperamental traits, separate ANOVAs were performed with temperament scales as dependent variables and socio-demographic characteristics as independent variables. Most of the sociodemographic factors (birth order, maternal and paternal age, maternal and paternal education, nationality, parental status, income) were not associated with the temperament scales scores, with the exception of gender, age, and socio-economical status. Specifically, for cortisol data boys had lower scores on Rhythmicity scale ($F$ = 5.03; $p$ = 0.03) than girls, whereas infants of low socio-economical status had higher scores on Threshold scale than infants of high socio-economical status ($F$ = 4.83; $p$ = 0.01); similarly, for alpha amylase data, boys had lower scores on Rhythmicity scale ($F$ = 4.99 ; $p$ = 0.03) and higher scores on Approach scale ($F$ = 5.13 ; $p$ = 0.03) than girls; younger infants (12-14 months) had lower scores on the Threshold scale ($F$ = 4.16 ; $p$ = 0.05) than older infants (15-18 months), whereas infants of low socio-economical status had higher scores on Threshold scale than infants of high socio-economical status ($F$ = 4.24; $p$ = 0.02). Consequently, gender, age, and socio-economical levels were considered as covariates in the following HMLM analyses.

Temperament and physiology

In order to test if temperamental traits predicted cortisol and A-A response to stress, separate HMLM analyses were conducted. For cortisol, "birth order", "time from awakening to assessment", and "duration of trip" - which were found to significantly predict cortisol data (see chapter 2) - were entered into the model jointly with temperament scales scores; similarly, for alpha amylase, "time from breakfast to assessment" and "medication" - which were found to significantly predict A-A data (see chapter 2) - were entered into the model together with temperament scales scores. Furthermore, gender and SES were also entered into HMLM models when Rhythmicity and Threshold were tested as predictors of cortisol data, whereas gender, age and SES were entered into HMLM models when Rhythmicity, Approach and Threshold were tested as predictors of alpha amylase data.

As shown in tables 4.5 and 4.6, the Adaptability scale scores significantly predicted cortisol concentrations, whereas Approach and Distractibility scales scores significantly predicted A-A concentrations.

Table 4.5 *Temperament scales scores as predictors of cortisol activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Activity** | -0.06 | 0.03 | -0.05 |
| Birth order | -0.65 | 2.19* | -1.54 |
| Time from awakening to assessment | -2.96** | 3.75*** | -3.09** |
| Duration of trip | 0.61 | 1.24 | -2.13* |
| **Rhythmicity** | 1.52 | -0.25 | -0.02 |
| Gender | -1.51 | -1.19 | 1.51 |
| Birth order | -0.17 | 2.32* | -1.80 |
| Time from awakening to assessment | -2.98** | 3.57*** | -2.92** |
| Duration of trip | 0.48 | 1.30 | -2.17* |
| **Approach** | 0.86 | 1.56 | -1.88t |
| Birth order | -0.78 | 2.14* | -1.46 |
| Time from awakening to assessment | -3.05** | 3.76*** | -3.10** |
| Duration of trip | 0.77 | 1.53 | -2.48** |
| **Adaptability** | 0.35 | **2.47**** | **-3.15**** |
| Birth order | -0.68 | 2.31* | -1.71 |
| Time from awakening to assessment | -2.97** | 3.33*** | -2.64** |
| Duration of trip | 0.60 | 1.08 | -2.05* |
| **Intensity** | -0.43 | 0.85 | -0.74 |
| Birth order | -0.63 | 2.20* | -1.55 |
| Time from awakening to assessment | -2.93** | 3.60*** | -2.94** |
| Duration of trip | 0.64 | 1.16 | -2.02* |
| **Mood** | 0.16 | 1.72 | -1.66 |
| Birth order | -0.65 | 2.03* | -1.37 |
| Time from awakening to assessment | -2.99** | 3.62*** | -2.95** |
| Duration of trip | 0.62 | 1.46 | -2.34* |
| **Persistence** | -0.43 | -0.31 | 0.46 |
| Birth order | -0.60 | 2.19* | -1.54 |
| Time from awakening to assessment | -2.93** | 3.79*** | -3.14** |
| Duration of trip | 0.49 | 1.12 | -1.93* |
| **Distractibility** | 1.00 | 0.80 | -1.23 |
| Birth order | -0.60 | 2.27* | -1.67 |
| Time from awakening to assessment | -2.65** | 3.75*** | -3.21** |
| Duration of trip | 0.61 | 1.22 | -2.11* |
| **Threshold** | 1.34 | 0.23 | -0.69 |
| SES | -0.24 | 0.02 | 0.21 |
| Birth order | -0.94 | 2.47** | -1.71 |
| Time from awakening to assessment | -3.31*** | 4.36*** | -3.28*** |
| Duration of trip | 0.20 | -0.23 | -0.21 |

The primary variables of interest for this study (temperament scales) are typed in bold, as well as their significant effect on cortisol data.

* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4.6 *Temperament scales scores as predictors of A-A activity. Results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Activity** | -0.02 | -0.04 | 0.51 |
| Time from breakfast to assessment | 0.50 | -2.38* | 2.23* |
| Medication | 0.46 | -2.19* | 2.13* |
| **Rhythmicity** | -0.73 | 0.21 | -0.06 |
| Gender | 1.16 | 0.21 | -0.13 |
| Time from breakfast to assessment | 0.59 | -2.37* | 2.27* |
| Medication | 0.40 | -2.21* | 2.13* |
| **Approach** | **-2.20*** | **2.31*** | **-2.04*** |
| Gender | 0.52 | 0.76 | -0.59 |
| Time from breakfast to assessment | 0.69 | -2.44* | 2.32* |
| Medication | 0.36 | -2.23* | 2.17* |
| **Adaptability** | -1.29 | 0.69 | -0.75 |
| Time from breakfast to assessment | 0.78 | -2.50* | 2.42* |
| Medication | 0.49 | -2.20* | 2.14* |
| **Intensity** | 0.39 | 1.33 | -1.50 |
| Time from breakfast to assessment | 0.34 | -2.76** | 2.71** |
| Medication | 0.52 | -2.03* | 1.95* |
| **Mood** | 0.38 | 1.20 | -1.56 |
| Time from breakfast to assessment | 0.42 | -2.65** | 2.60** |
| Medication | 0.43 | -2.32* | 2.29* |
| **Persistence** | -0.36 | 0.72 | -0.93 |
| Time from breakfast to assessment | 0.48 | -2.41* | 2.31* |
| Medication | 0.49 | -2.25* | 2.22* |
| **Distractibility** | -0.32 | **2.88**** | **-2.98**** |
| Time from breakfast to assessment | 0.44 | -2.30* | 2.23* |
| Medication | 0.49 | -2.67** | 2.65** |
| **Threshold** | -0.29 | 0.51 | -0.35 |
| Age | 0.45 | -0.32 | 0.13 |
| SES | -0.29 | 0.56 | -0.47 |
| Time from breakfast to assessment | 0.49 | -2.25* | 1.84 |
| Medication | 0.39 | -2.12* | 2.02 |

The primary variables of interest for this study (temperament scales) are typed in bold, as well as their significant effect on alpha amylase data.
p<0.05; ** p < 0.01; *** p < 0.001

The direction of the effect of Adaptability on cortisol is displayed in figure 4.3. Specifically, infants with low scores on Adaptability scale had a sharp decrease at 20 minutes followed by an increase at 40 minutes after the stressful procedure in comparison with infants with high scores who showed more stable cortisol levels across the three time collections.

To test whether infants with low scores on Adaptability scale differed along another temperamental trait associated with low proneness to distress (infants classified as A1-B2) previously investigated (see chapter 3), one way ANOVA with A1-B2 vs B3-C2 groups as independent variable and Adaptability scale scores as dependent variable was performed. Infants with different proneness to distress did not show significantly different mean scores on this scale ($F = 1.13$; $p = 0.29$).

The direction of the effects related to Approach and Distractibility scales scores on A-A activity is displayed in figure 4.4. Infants with high scores on Approach had lower alpha amylase baseline levels but showed a greater increase of A-A from pre to 20 minutes post the strange situation compared with infants with low scores on this scale (Panel A). Similarly, infants with high scores on Distractibility showed a significant increase of alpha amylase concentrations 20 minutes post the strange situation in respect with the baseline compared to infants with low scores on this scale (Panel B).

Figure 4.3
*Cortisol levels (in µg/dl) predicted by Adaptability scale scores. Results of the HMLM analyses*

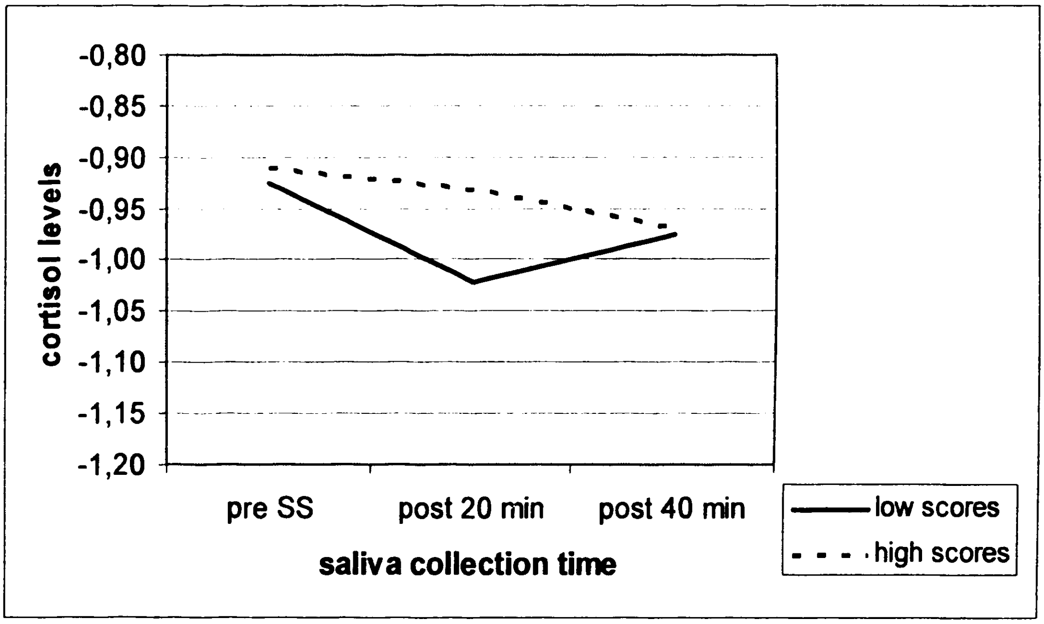
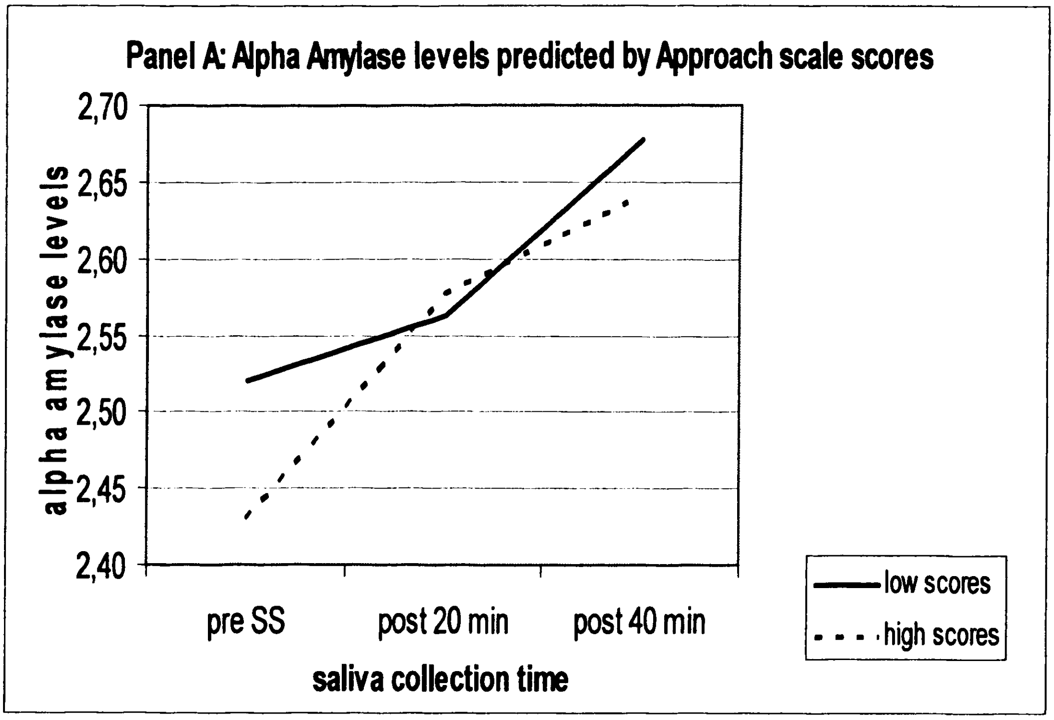
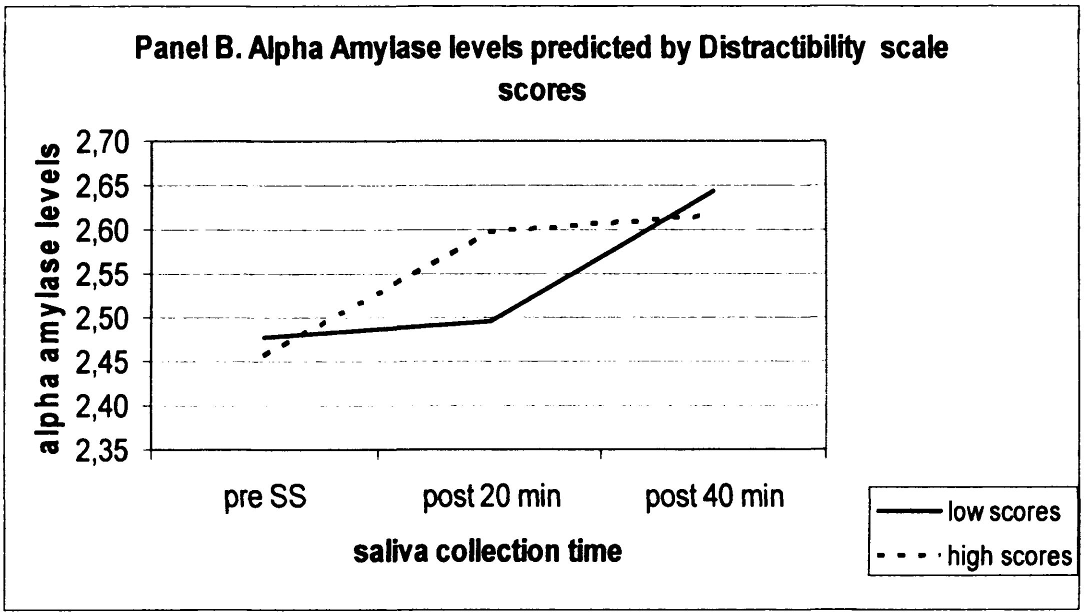



Figure 4.4
*Alpha amylase levels (in U/mL) predicted by Approach and Distractibility scales scores. Results of the HMLM analyses*






In order to test whether Approach and Distractibility scales had significantly independent effects on A-A levels, they were entered together into the HMLM model. As shown in table 4.7, A-A levels were found to be predicted by both scale scores. Moreover, separate ANOVAs with proneness to distress (A1-B2 vs B3-C) as independent variable and Approach and Distractibility scale scores as dependent variables revealed a significant association between proneness to distress and the Approach scale scores, but no effect on Distractibility scores. Specifically, infants classified as B3-C had higher mean scores on Approach scale than infants classified as A1-B2 ($F = 3.10; p = 0.02$).

Table 4.7

*Temperament scales scores as predictors of alpha amylase activity. Final results of the hierarchical linear model*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Approach** | **-2.25*** | **2.17*** | **-1.95*** |
| **Distractibility** | -0.03 | **2.85**** | **-2.85**** |
| Gender | 0.42 | 1.13 | -1.05 |
| Time from breakfast to assessment | 0.45 | -2.26* | 2.04* |
| Medication | 0.23 | -2.77** | 2.66** |

The primary variables of interest for this study (temperament scales) are typed in bold, as well as their significant effect on alpha amylase data.

* p<0.05; ** p < 0.01; *** p < 0.001

# Discussion

The present study provided a first contribution to the investigation of the effects of genes and temperamental traits in predicting HPA and SAM activity, as indexed by salivary cortisol and alpha amylase activity, in infancy.

*Genetics and physiology*

Although there is a large consensus that genetics may explain individual differences in reaction to stressful events, this is the first study which has investigated this issue in infancy. Moreover, no studies have looked into genetic contributors to alpha amylase response to challenging circumstances.

A major finding of the present study was an association between the - 521 C/T single nucleotide polymorphism (SNP) in the DRD4 promoter region and alpha amylase levels. Specifically, infants with the CC variant had higher alpha amylase levels across the three saliva collections than infants with the CT and TT variants, suggesting a contribution of dopamine D4 receptor availability to individual differences in alpha amylase levels. However, the interaction of the exon III 48-bp repeat and the – 521 C/T promoter polymorphisms of the DRD4 gene did not predict alpha amylase levels, as might have been expected from the results of Lakatos and colleagues (2002). Lakatos et al. (2002) found that the presence of -521 T allele, in combination with the 7 repeat allele of the DRD4 gene increased the risk of developing disorganized attachment in infants (Lakatos et al., 2002). Nevertheless, other studies have shown a significant effect of the DRD4/521 gene alone on some temperamental traits and in specific clinical populations. Specifically, a significant association between the CC genotype of -521 SNP and the human personality trait of Novelty Seeking (a human personality trait characterized by impulsive, exploratory, or sensation-seeking behaviour) has been found in Japanese and Hungarian samples of adult participants (Okuyama et al., 2000; Ronai et al., 2001), and the C variant has been documented to be more common among schizophrenic patients (Okuyama et al., 1999). Further studies are needed to evaluate the contribution of the DRD4 gene to individual differences in alpha amylase response, taking also into account its possible role in combination with other genetic and environmental factors.

The other polymorphisms of the 5-HTT and COMT genes, thought to have a direct influence on stress response given their role in metabolism of the catecholamines, as well as the DRD4 gene were not found to predict cortisol and alpha amylase levels in response to the Strange Situation Procedure. However, an interesting trend in relation to cortisol reaction to stress emerged when 5-HTT genotype was grouped into SS versus SL and LL allele carriers. In particular, infants with the SS allele had higher cortisol baseline levels and showed a significant decrease in this hormone after 20 minutes from the end of the stressful procedure, followed by a slight increase after 40 minutes compared to infants with LL and SL allele, who had largely invariable cortisol levels across the three saliva collections. Although the effect was modest ($p = 0.07$), this finding supports the role of LL and SL genotypes as protective factors against stress in line with studies carried out with animals and humans (Caspi et al., 2003; Lanfumey et al., 2000; Li et al., 1999; Wichems et al., 2000). Further studies should better clarify if individuals with the SS genotype are at higher risk of anomalous HPA axis functioning as indexed by heightened cortisol baseline levels.

To conclude, the present findings should be interpreted keeping in mind some limitations. First, it is plausible that a relatively large number of genetic polymorphisms would be responsible, additively or interactively, for a substantial part of variance in stress response in infancy. In fact, similar to other complex or non-Mendelian disorders, many genes appear to be involved in the functioning of the multifaceted HPA and SAM systems. Second, the small sample size limits the robustness of the results and might mask the effects of some polymorphisms; research employing larger samples will be required to more clearly look into the nature of the associations found in the present investigation.

*Temperament and physiology*

The findings of this study showed that some individual differences in temperament as assessed by maternal reports were associated with salivary cortisol and alpha amylase levels in response to the Strange Situation procedure.

Considering HPA axis functioning first, infants who displayed relative difficulty adjusting to change (e.g. protests towards changes in schedules of meals/sleep/bath), as assessed by adaptability scale scores, showed a less marked cortisol decreasing from pre to post Strange Situation compared to more adaptable infants, who showed a more marked cortisol decreasing from pre to 20 minutes post SS followed by a slight cortisol increasing 40 minutes post SS. Thus, although both groups showed no glucorticoids increase in response to stress, less adaptable infants displayed a less evident decrease of cortisol concentrations after the stressor. Taking into account the effect of cortisol diurnal decline found in the present study (see chapter 2), it is likely that such a decline may have masked minor cortisol increases actually triggered by this temperamental trait. However, the only study (Wilson et al., 2003) which has examined the relation between adaptability and HPA axis response to immunizations in a small sample of 2 and 4 months infants did not find any significant correlation.

Furthermore, the current study did not replicate the correlation between adrenocortical hyperactivity and one of the most investigated temperament traits, namely behavioural inhibition, found in some studies (Kagan et al., 1987; Flinn and England, 1997; Schmidt et al., 1997). In fact, infants with high scores on Approach scale, often measured by other researchers to assess behavioural inhibition, did not have higher pre and post SS cortisol levels than infants with low scores on this scale. However, other studies did not corroborate the direct association between behavioural inhibition and cortisol reactivity, but found that other social and contextual factors (i.e. attachment relationship) may act as moderators of the relation between temperament and adrenocortical activity (Nachmias et al., 1996; Schieche and Spangler, 2005; Spangler and Schieche, 1998; van Bakel and Riksen-Walraven, 2004). Thus it is possible that even in the present study the effect of behavioural inhibition on HPA axis functioning may be better understood in combination with other factors.

In respect to sympatoadrenomedulla system activity, alpha amylase changes in this study were related to the temperament characteristics of approach and distractibility. Infants who were assessed as behaviourally inhibited (i.e. scored high on approach) by their mothers displayed alpha amylase reactivity from pre to 20 minutes post-stressor compared to infants who were not behaviourally inhibited in line with other studies (El Sheikh and colleagues, *under review*; Scarpa et al., 1997). Moreover, infants who were classified as highly prone to distress (B3-C) had higher scores on Approach scale, thus confirming how these temperamental traits are sides of the same coin which can shape individual differences in infant's stress response. Last, infants who had high scores on the Distractibility scale displayed an increase of alpha amylase levels from pre to 20 minutes post the stressful procedure compared to infants who had low scores on this scale. As having high scores on Distractibility indicates an inability of infants to be easily distracted from external stimuli and thus possibly not being easily reassured, this finding supports the relation between this "difficult" temperamental trait and a fast hyper-activation (within 20 minutes) of the sympatoadrenomedulla system. This result converges with that of Wilson and colleagues (2003), who reported a significant association between distractibility and cortisol changes in response to the stress of inoculations.

To sum up, some temperament traits were found to be associated with differences in cortisol and alpha amylase activity in response to a mild stressor. However, each physiological system was associated with differing aspects of temperament, a finding consistent with the frequently-reported independence of these physiological markers of stress. Dissociations are common in psychobiological studies and might partly be explained by the fact that psychobiological responses to threatening, stressful situations load on two relatively distinct factors (see Lundberg and Frankenhaueser, 1980; Ursin et al, 1978), namely effort and distress. The effort factor is significantly associated with vigilance, attention, and involvement, whereas the distress factor has high loadings on feelings of helplessness, loss of control, fear and anxiety. Catecholamine activity and related cardiac measures tend to load on the first factor, while cortisol tends to load highly on the second factor. Therefore, individual differences in temperament might be differentially associated with cortisol and alpha amylase responses because of the activation of distress and effort components respectively. Nevertheless, in the light of the obvious limitation represented by the use of a questionnaire rather than more reliable observational measures to assess infant's temperament in this study, any interpretation of present data is just speculative.

Although the current study found some genes and temperamental traits to be predictive of HPA axis and SAM system activity, further studies aimed at investigating the effects of the interaction between biological and environmental factors on salivary cortisol and alpha amylase levels could contribute to better understand the multifaceted phenomena of stress response in infancy. This issue will be the object of the next chapter.

# CHAPTER 5

# THE ROLE PLAYED BY THE INTERACTION BETWEEN BIOLOGICAL FACTORS AND ATTACHMENT IN THE STRESS RESPONSE

## Introduction

In the last decade, there has been a growing interest in the interdependence of biological and environmental influences in affecting behaviour and psychopathology. Given the great attention paid to gene-environment interplay (Moffitt et al., 2005), several psychobiological studies have focused on the effects of the interaction between specific genetic polymorphisms and different environmental factors on behavioural phenotypes. However, to what extent gene-environment interplay predicts individual differences in physiological stress responses in infancy, as indexed by salivary cortisol and alpha amylase levels, is still unknown. Instead, there is some evidence regarding the role played by temperament (considered to be largely determined by biological factors) when combined with the quality of maternal care (i.e attachment relationship) in determining hypothalamic-pituitary-adrenal (HPA) axis activation after a stressor in childhood. As already written in the previous chapters, the determinants of individual differences in alpha amylase response to stress are largely unidentified, and consequently no study has investigated the effect of temperament-attachment interaction on this saliva biomarker. However, some studies have looked into other markers of the autonomic nervous system activity, such as respiratory sinus arrhythmia, heart rate and pre ejection period, as a function of the interaction between temperament and attachment (Stevenson-Hinde and Marshall, 1995; Oosterman and Schuegel, 2007).

In the following sections, studies on the interaction between the genetic polymorphisms investigated in the present study and environmental factors, as well as between temperamental traits and attachment as possible contributors of physiological stress response in childhood will be reviewed. This is followed by a summary of the rationale and hypotheses of the current study.

*Gene-Environment interaction*

It is widely recognized that genetic effects may be expressed more strongly under some environmental conditions than others, and environmental effects may be conditional on certain genotypes. Also, exposure to specific environments may be influenced by the individual's genetic make-up (for a review, see Rutter et al., 2006). In relation to developmental science, the pioneering studies of Michael Meaney and his colleagues, using rats to investigate the effects of early rearing experiences, provide arguably the best examples of how environments can have effects on gene expression (Cameron et al., 2005; Champagne et al., 2004; Meaney and Szyf, 2005; Weaver et al., 2004). The starting point for their work was the observation that lactating mother rats varied markedly in the extent to which they licked and groomed their neonatal offspring, showing arched back nursing, and that variation in these maternal behaviours was associated with individual differences in the offspring's behaviour and response to stress. A cross-fostering design clearly showed that this intergenerational influence was a function of rearing and not biological parentage. It was found that the behavioural and psychophysiological consequences were mediated by DNA methylation effects on a specific glucocorticoid receptor gene promoter in the hippocampus. Moreover, it was demonstrated that a drug called trichostatin-A could reverse the methylation effect and that this did indeed change the endocrine response to stress.

Similarly to the work of Meaney's group, Suomi and colleagues have been investigating the interactions between genes and environment in predicting behaviour and physiological stress response in nonhuman primates (Barr et al., 2004; Barr et al., 2003; Champoux et al., 2002; Suomi, 2006). The authors found that monkeys carrying the short allele of the serotonin transporter gene show delayed early neurobiological development, impaired serotoninergic functioning, and excessive aggression, HPA reactivity, and alcohol consumption only if they were reared with peers (Suomi, 2006). Although further studies are needed in order to understand how far these findings can be generalized to humans, an increasing number of investigations have been studying the complex interplay between genes and environment in the field of developmental psychology.

To date, the effects of some polymorphisms in combination with environmental factors on different behavioural dimensions have been investigated, although no study has investigated such interactive effects on psychophysiological stress reactivity.

In humans, two common VNTR alleles of the promoter region of the serotonin transporter gene (5-HTTLPR), one short and one long, have been differentially associated with anxiety-related behavioural traits in healthy subjects (Lesch et al., 1996; Lesch and Mossner 1998). At the behavioural level, individuals carrying the short allele are slightly more likely to display abnormal levels of anxiety (Lesch et al., 1996; Katsuragi et al., 1999; Melke et al., 2001), acquire conditioned fear responses (Garpenstrand et al., 2001), and develop affective illness (Lesch and Mossner, 1998) compared with those homozygous for the long allele. Further, studies in adults and children have implicated 5-HTT in gene-environment interactions. In adults, individuals with two copies of the short allele exhibited major depressive disorder in relation to stressful life events than individuals homozygous for the long allele (Caspi et al., 2003). In children, Kaufman and colleagues (2004) found that maltreated children with the s/s genotype and no positive supports had the highest depression ratings scores, that were twice as high as the non-maltreated comparison children with the same genotype; Fox et al (2005) found that children with the combination of the short 5-HTT allele and maternal reports of low social support had increased risk for behavioural inhibition in middle childhood.

The dopamine D4 receptor (DRD4) gene with variable numbers of a 48 bp repeat (48 bp VNTR) in exon III is one of the most frequently studied candidate genes in psychobiological studies. The 7-repeat variant of the dopamine D4 receptor is two to three times less potent in dopamine-mediated coupling to adenylyl cyclase than the most common 4-repeat form and it has been associated with pathological impulsive behaviour and substance abuse in adults, and ADHD in children. Further, recent studies have found this polymorphism to be involved in gene-environment interactions. Bakermans-Kranenburg and Van IJzendoorn (2006) found a sixfold increase in externalizing behaviours in preschool children with the 7-repeat allele exposed to insensitive care compared to children without this combination of risk factors. Moreover, two recent studies have investigated DRD4-environment interaction in the context of attachment research on infant disorganization. A first study examined the interaction between genetic (DRD4 7-repeat and –521 C/T) and environmental risk factors (maternal state of mind in respect to attachment, and frightening behaviour) in predicting infant disorganization (Van IJzendoorn and

Bakermans-Kranenburg, 2006). The results of this study suggested that the DRD4 7-repeat allele elevates the risk for infants becoming disorganized, but only in the presence of a distal environmental risk factor, namely maternal unresolved state of mind as assessed by the Adult Attachment Interview (George, Kaplan, Main, 1996). The presence of a proximal risk factor, namely maternal frightening behaviour as assessed by the Frightening Scale (Main and Hesse, 1992/1995), was not associated with an increased risk of developing disorganized attachment in infants with the long DRD4 variant, as well as the -521 C/T promoter gene did not play a significant role. In a collaborative study (Gervai et al., In Press) between Lyons-Ruth's group in USA and Gervai's group in Hungary, the interplay between genetic and caregiving contributions to disorganized attachment in 138 mother-infants dyads was investigated again. The authors found a strong relation between maternal disrupted communication and infant disorganization which was moderated by the infant's DRD4 7- repeat genotype. Specifically, attachment disorganization was only strongly associated with the quality of mother-infant communication in the absence of 7 allele. On the contrary, the 7 - repeat allele appeared to silent the infant's sensitivity to care, acting as a protective factor in the context of very disrupted interactions but providing less regulation in the context of higher quality of care. Although the results of these investigations show interesting differences in how the interaction between DRD4 genotype and proximal versus distal environmental risk factors predict disorganized attachment, both studies support the role played by genetic vulnerability, which in combination with an environmental risk factor may lead to attachment disorganization.

The catechol O-methyltransferase (COMT) gene contains a common, functional polymorphism in which a val-to-met amino acid substitution markedly reduces enzyme activity in catabolizing dopamine, epinephrine, and norepinephrine to about 20% of wild-type (val) levels (Lachman et al., 1996; Lotta et al., 1995). Recent studies have suggested a role for COMT in behaviours associated with impulsivity and disinhibition (Lachman et al., 1998; Kotler et al., 1999; Strous et al., 1997), emotional and pain response (Smolka et al., 2005; Zubieta et al., 2003a), cortical response to the performance of cognitively demanding tasks (Goldberg and Weinberger, 2004), response to antidepressive treatment (Szegedi et al., 2005) and the risk for several neuropsychiatric conditions (Craddock et al., 2006; Karayiorgou et al., 1999).

Recent reports support the possibility of gene–environment interactions involving the COMT Val/Met polymorphism. In a longitudinal study of the Dunedin birth cohort followed to adulthood, carriers of the COMT Val allele were most likely to exhibit psychotic symptoms and to develop schizophreniform disorder if they used cannabis, whereas cannabis use had no such adverse influence on individuals with two copies of the Met Allele (Caspi et al., 2005). Further, in a family-based association study of 240 ADHD sufferers, bearers of the Val/Val genotype were substantially more susceptible to the adverse effects of prenatal risk (indexed by low birth weight) in influencing risk of early-onset antisocial behaviour (Thapar et al., 2005). Last, Jabbi et al (2007) found significant interactions between COMT and diagnostic group (patients with major depressive disorder, healthy high risk probands to MDD and healthy controls) in measures of plasma epinephrine, cortisol and subjective responses to a laboratory stress test in adults. Specifically, a higher epinephrine response during stress and a higher overall cortisol and epinephrine responses were observed as a function of *met* allelic loading in the healthy controls, whereas the healthy high risk probands (unaffected family members of depressed probands) showed a higher endocrine stress response in those homozygous for the *val* allele compared with other genotypes; last, in depressed patients individuals homozygous for the met allele tended to show higher epinephrine response during the stressful task compared with other genotypes, although no differences in overall cortisol response was found between genotypes.

To sum up, although different studies have reported a significant role of some polymorphisms in predicting behavioural traits when combined with some environmental factors, there are no data on whether gene-environment interactions predict individual differences in physiological stress response in human infants.

*Temperament-attachment interaction*

Earlier studies have suggested that HPA axis reactivity in infants may be related to temperamental characteristics (Gunnar et al., 1996; Gunnar et al., 1992; Nachmias et al., 1996), and to attachment security (Gunnar et al., 1996; Hertsgaard et al., 1995; Spangler and Grossman, 1993; Spangler and Schieche, 1998).

With regard to temperamental characteristics, on the basis of the neural circuitry involved in activating stress responses of both the HPA axis and the sympathetic adrenomedullary (SAM) system, most empirical attention has been focused on inhibited or fearful behaviours. It has been suggested that these temperamental traits, in the extreme, may reflect a lowering of the threshold for activation of stress-sensitive physiological systems (Tout et al., 1998). However, the relationship between stress response and fearfulness has not consistently been found to be in line with theoretical assumptions. In fact, although several studies found fearfulness to be positively associated with elevated cortisol levels (Nachmias et al., 1996; Kagan et al., 1987; Schmidt et al., 1997; Watamura et al., 2002), as well as with high and stable heart rate and right frontal EEG activation (Calkins et al., 1996; Fox et al., 1995; Marshall and Stevenson-Hinde, 1998) in infancy and childhood, others did not (de Haan et al., 1998; Gunnar et al., 1992; Gunnar et al., 1997; Marshall and Stevenson-Hinde, 2001, Oosterman and Schuengel, 2007). Further, research is now suggesting that the other end of the socially reactive spectrum, boldness or surgency, is also associated with differences in physiological activity. Several studies have reported a relationship between boldness and increased cortisol levels in response to different social challenges in preschool and elementary children (e.g., Davis et al., 1999; Donzella et al., 2000; Flinn, 1999; Granger et al., 1994; Gunnar et al., 1997), whereas the literature concerning autonomic underarousal and behaviour problems indicates that high heart period (slow heart rate) has been associated with externalizing behaviours in children (e.g., van Goozen et al., 1998).

Given these inconsistent results, it is possible that the relationship between some temperamental traits and the activation of physiological stress systems can be better explained if other factors are taken into account. Among these factors, researchers have investigated the ways in which infants cope with stress, focusing on child-mother attachment relationship as a significant behavioural coping resource for the child against stress. In fact, as well as temperamental factors, parenting behaviours and parent–child relationships are likewise considered significant forces in determining the psychophysiological response to stress (e.g. Gunnar and Donzella, 2002). Several findings from biobehavioural studies indicate that during emotional challenges the behavioural strategy of securely attached infants is more adaptive, as adrenocortical activation was observed only in insecurely attached or disorganized infants and not in securely attached ones (Hertsgaard et al., 1995; Spangler and Grossman, 1993; Spangler and Schieche, 1998).

Moreover, attachment security has been found to moderate the relation between some temperament traits and physiological stress response in infancy and early childhood. Specifically, several studies have suggested that a secure attachment relationship functions as a social buffer against less adaptive temperamental dispositions in different stressful circumstances. Nachmias and colleagues (1996) were the firsts to investigate the interplay between infant attachment and behavioural inhibition in cortisol response to the strange situation and to another observational procedure devised for measuring toddler inhibition of approach to novel events. In this study the authors tested the hypothesis that behavioural inhibition in the face of novel, arousing events was associated with cortisol elevations only for children who were insecurely attached. They found that insecure, higher-inhibition infants had higher post-session cortisol levels than secure, higher inhibition children. Another study published two years later (Spangler and Schieche, 1998) supported this finding in a larger sample of infants, whose quality of attachment, emotional expression, and adrenocortical reactivity were assessed. In particular, the authors reported that insecure ambivalent infants characterized by high proneness to distress showed adrenocortical activation in response to the strange situation in comparison to secure infants similarly characterized by high proneness to distress (B3 and B4) according to the Belsky and Rovine (1987) classification. Moreover, they also found that behaviourally inhibited infants (as assessed by maternal report) with an insecure attachment relationship exhibited adrenocortical activation in contrast to behaviourally inhibited infants with a secure relationship. In another study, the same authors (Schieche and Spangler, 2005) demonstrated that secure attachment functions as a social buffer against HPA axis reactivity in infants with high behavioural inhibition also in a primarily non-attachment-related- challenging context. Specifically they found adrenocortical activation only in insecure infants with high behavioural inhibition. However, although there is some evidence that attachment security is a protective factor against HPA reaction in inhibited infants, a recent investigation (van Bakel and Riksen-Walraven, 2004) did not support the role played by attachment security as a moderating factor between unfavourable behavioural traits (social fearfulness) and cortisol reactivity.

Similarly, other studies have investigated the activity of the autonomic nervous system in relation to behavioural inhibition and attachment. Stevenson-Hinde and Marshall (1999) assessed the relation between autonomic functioning and both behavioural inhibition and attachment status in a sample of 4.5-year olds children who were administered a modified version of the Strange Situation, predicting that high heart period and high respiratory sinus arrhythmia would be related to low behavioural inhibition when attachment status was taken into account. The main finding of the study confirmed that the predicted relation between cardiac functioning and behavioural inhibition was found only in securely attached children, whereas the respiratory sinus arrhythmia results were not statistically significant. However, a recent study of Burgess and colleagues (2003) found that both early temperament and early attachment independently but not interactively contributed to subsequent physiological and behavioural functioning. Specifically, the authors assessed attachment classification at 14 months of age, behavioural inhibition at 24 months, and relevant physiological measures (heart rate and sinus arrhythmia) at 4 years of age in a sample of 140 children. They found that children with an avoidant attachment relationship displayed significantly higher heart period (lower heart rate) and higher respiratory sinus arrhythmia than secure and anxious-resistant children at 4 years of age but not at 14 months and at 2 years.

Last, as far as we know, no studies have examined alpha amylase reactivity to stress in infants as a function of the interaction between temperamental traits and attachment status; moreover, also the relation between other temperamental characteristics (different from behavioural inhibition) and attachment relationship in predicting physiological stress response has not been investigated yet.

The current study is aimed at examining the interaction between infant internal characteristics (genes and temperament) and environment (attachment relationship) in determining the stressfulness of separation. Specifically, the main objectives were to test the hypotheses that physiological response to stress in infancy are predicted by attachment quality in combination with 1) genetic polymorphisms, and 2) infant's temperament characteristics. Before answering the research questions, a set of preliminary analyses were run in order to test if attachment status was associated with genes and temperament.

## Methods

*Participants and design*

The data came from an initial sample of 82 healthy, middle class, 12 to 18–month old (mean age = 14.6; SD = 1.8) infants (45 boys) and their mothers who agreed to participate in the study and gave their signed informed consent. To index environmental and genetic effects on infants' physiological responses to stress, attachment classifications, a maternal report of infant's temperament, genotyping (5-HTT, DRD4, DRD4/521, COMT) status along with salivary cortisol and alpha amylase concentrations were assessed. 8 and 6 infants were not included in the present analyses because they did not have any cortisol and alpha amylase data respectively, 2 infants were excluded because their mothers did not fill in the Toddler Temperament Scale (Fullard et al., 1984), and genotyping of DRD4, DRD4/521, 5-HTTLPR and COMT was not respectively successful for 5, 2, 4 and 1 infants. Table 5.1 shows the main demographic characteristics, attachment patterns and genotype distribution of the final samples. On the measures of attachment patterns, genotyping status, temperament traits, main demographic characteristics (gender, age, birth order, maternal education and age, SES) and infant's state factors (related to sleep, food, physical conditions and daily experiences) participants did not differ from the remaining infants with incomplete data with the exception of some variables related to sleep, namely "hours of sleep the night before the assessment", "time of wake up" and "minutes from awake to saliva collection" .

To control for diurnal variation of cortisol levels, parent-infant dyads were assessed in one morning session at the Psychopathology Unit of the "E.Medea" Scientific Institute. Upon arrival, the mothers were given a brief overview of the session, a brief sociodemographic form as well as a form related to potentially interfering factors (time of assessment, the infant's health, mood, sleep, feeding, medications and duration of the trip to the lab) on salivary physiological data were filled in (see methods section of chapter 2 for a full description of both forms) while a saliva sample was collected from the infant. The mother and infant then participated in the Strange Situation procedure in a different testing room. Infant's saliva was collected again 20 and 40 minutes from the end of the procedure while mother completed the Toddler Temperament Scale. Finally, genomic DNA was collected with buccal swabs. The Ethical Committee of University College London and "Eugenio Medea" Scientific Institute approved the research protocol.

Table 5.1
*Sample characteristics*

| | *Cortisol data* | | *Alpha amylase data* | |
|---|---|---|---|---|
| | *f* | *%* | *f* | *%* |
| **MAIN CHILD'S DEMOGRAPHIC CHARACTERISTICS** | | | | |
| **Gender** | | | | |
| *Boys* | 38 | 52.8 | 39 | 52.7 |
| *Girls* | 34 | 47.2 | 35 | 47.3 |
| **Age** | | | | |
| *12-14 months* | 36 | 50 | 36 | 48.6 |
| *15-18 months* | 36 | 50 | 38 | 51.4 |
| **Ses** | | | | |
| *Low* | 4 | 5.7 | 4 | 5.6 |
| *Medium* | 31 | 44.3 | 32 | 44.4 |
| *High* | 35 | 50 | 36 | 50 |
| **ATTACHMENT** | | | | |
| *Secure* | 37 | 51.4 | 38 | 51.4 |
| *Avoidant* | 10 | 13.9 | 10 | 13.5 |
| *Resistant* | 12 | 16.7 | 12 | 16.2 |
| *Disorganized* | 13 | 18.1 | 14 | 18.9 |
| **Security of attachment** | | | | |
| *Secure* | 37 | 51.4 | 38 | 51.4 |
| *Insecure* | 35 | 48.6 | 36 | 48.6 |
| **GENES** | | | | |
| ***5-HTT*** | | | | |
| No risk (LL and SL) | 56 | 78.9 | 58 | 79.5 |
| At risk (SS) | 15 | 21.1 | 15 | 20.5 |
| ***DRD4*** | | | | |
| No risk (-7 allele) | 48 | 69.6 | 50 | 70.4 |
| At risk (+ 7 allele) | 21 | 30.4 | 21 | 29.6 |
| ***DRD4/521*** | | | | |
| No risk (CC variant) | 22 | 30.6 | 22 | 29.7 |
| At risk (CT and TT variants) | 50 | 69.4 | 52 | 70.3 |
| ***COMT*** | | | | |
| No risk (GG variant) | 21 | 28.8 | 22 | 29.3 |
| At risk (AA and GA variants) | 52 | 71.2 | 53 | 70.7 |

*Instruments*

**Strange Situation.** The Strange Situation (SS) is a 20-min situation involving a sequence of episodes, which progressively activate the attachment system—entrance into an unfamiliar environment, the arrival of a stranger, two brief separations from the mother, and two subsequent reunions with her (Ainsworth et al., 1978). All Strange Situation were videotaped and subsequently coded for A (Anxious-Avoidant), B (secure), and C (Anxious/Resistant) attachment classifications according to procedures described in Ainsworth et al. (1978). Coding of the D classifications followed the procedures described in Main and Solomon (1990).

Infants scoring 1 through 4 on the D scales were classified as non-Disorganized (non-D), while those scoring 5 through 9 were classified as Disorganized (D). The main coder was trained by Alan Sroufe for the A, B and C attachment categories and by Elisabeth Carlson for the D classification. Another researcher of the IRCCS E.Medea trained by the main coder for ABC classifications rated a subsample of videotapes; the intercoder reliability established on the basis of 29 cases was found to be 82.7% (Cohen's Kappa = .74). Attachment classification was also coded in terms of the A1-B2 versus B3-C2 framework. In the original sample, 43 (52,4%) infants were classified as secure, 10 (12,2%) infants as avoidant, 14 (17,1%) infants as anxious-resistant, and 15 (18,3%) infants as disorganized. In this study, infants were grouped into secure versus insecure categories because of the relatively small numbers in each insecure category.

For a full description of the Strange Situation procedure, see methods section of chapter 3.

**The Toddler Temperament Scale.** The Toddler Temperament Scale (Fullard et al., 1984) is a 92-item caretaker questionnaire, which are evaluated on a Likert scale, designed to assess the nine temperamental dimensions as described by Thomas and Chess (1977). The nine temperament characteristics measured by the questionnaire are activity, rhythmicity, approach/withdrawal, adaptability, intensity, mood, persistence/attention span, distractibility, and sensory threshold. High scores on each scale are indicative of "difficult" temperament traits. The Italian version of the questionnaire has been translated and validated by Axia (1993). All temperament scale scores were normally distributed.

For a full description of the Toddler Temperament Scale, see methods section of chapter 4.

*Psychophysiological assessment*

**Salivary Cortisol /Alpha Amylase Collection and Assay.** Cortisol and alpha amylase responses were assessed by infant's saliva, using a sterile cotton roll lightly dusted on the tip with 2 or 3 grains of Kool Aid crystals or Sorbette (Salimetrics). Saliva samples were then stored at – 80° C until the assay. Two competitive immunoassays (HS-Cortisol EIA Kit and Expanded Range (ER) HS-Cortisol EIA Kit, Salimetrics) specifically designed for the quantitative measurement of salivary cortisol were used for the assessment of cortisol in the biology lab of the "E.Medea" IRCCS. Samples were run in duplicate whenever possible, and all samples from one infant were run in the same assay to minimize method variability. A commercially available kinetic reaction assay (Salimetrics, State College, PA) was used for the assessment of alpha amylase levels in the Salimetrics lab (University of Pennsylvania) where the saliva samples were shipped to on dry ice.

Examination of the salivary cortisol and A-A data revealed that they were not normally distributed. Log transformations (cortisol and alpha amylase data were $\log_{10}$ and $\log_{10} + 1$ transformed respectively) were used to establish normal distributions. Of the original 74 participants with cortisol data, one was excluded as outlier beyond 3 SD above the mean for all cortisol data. No effects of saliva collection methods (rope versus sorbette), cortisol assay (duplicate versus singlet) and cortisol kits on cortisol levels were found, as well as no effect of saliva collection method (rope versus sorbette) on alpha amylase levels emerged.
For a full description of salivary biomarkers collection and assay, see methods section of chapter 2.

*Genetic Assessment*

**DNA preparation and genotyping.** Genomic DNA was collected by buccal swab and extracted with the DNAzol genomic DNA isolation reagent (MRC, Cincinnati, OH). The four genetic polymorphisms (DRD4, DRD4/521, COMT and 5-HTT) investigated in the present study were analyzed by polymerase chain reaction (PCR) amplification and agarose gel electrophoresis in the Molecular Biology laboratory at the "Eugenio Medea" Scientific Institute.

All the genotyping were done blind to all the other variables (i.e. attachment status, temperament, physiological outcomes). For statistical purposes, the genotypes were grouped by the absence or presence of the 7-repeat allele for the DRD4, into CC and T (encompassing T and C/T) alleles for the DRD4/521, into AA and G (encompassing GG and GA) alleles for the COMT, and into SS and L (encompassing LL and LS) alleles for the 5-HTT whereas one SI allele carrier infant was excluded from the analyses. All genotypes were in Hardy-Weinberg equilibrium with the exception of the DRD4/521.
For a full description of genetic assay and genotyping, see methods section of chapter 4.

# Results

In this section, the effects of gene-attachment interaction as well as temperament-attachment interaction on salivary cortisol and alpha amylase activity will be tested by hierarchical multivariate linear model analyses (HMLM). Preliminarily, the associations between genetic polymorphisms and attachment status, and between temperament and attachment status will be investigated. Then, only the significant effects of the interactions which were found to predict HPA and SAM activity will be shown for sake of space. In order to avoid redundancy, the independent effects of genes, temperamental traits and attachment status on stress-related physiological functioning will be displayed in the tables but not presented in the text just in case they were not emerged in previous chapters.

Findings about genes x attachment and temperament x attachment interactions will be reported separately.

*Genes – attachment interactions on physiological response to stress*

To control for the presence of associations between genetic polymorphisms and attachment classification chi-square analyses were performed. As shown in table 5.2, no significant associations were found between each polymorphism (COMT, DRD4, DRD4/521 and 5-HTT) and attachment security (secure vs insecure). However, when all 4 attachment groups were considered, a significant association emerged between DRD4/521 polymorphism and attachment classification ($\chi^2$ (6) = 12.54; $p$ = 0.05); specifically, infants with disorganized attachment were more likely to have CT genotype (standardized residuals = 1.96).

Table 5.2

*The distribution of participants by attachment classification and genetic polymorphisms*

| | Attachment patterns | | | | p | Security of attachment | | p |
|---|---|---|---|---|---|---|---|---|
| | B | C | A | D | | Secure | Insecure | |
| ***5-HTT*** | | | | | Ns | | | Ns |
| LL | 15 | 6 | 2 | 7 | | 15 | 15 | |
| LS | 19 | 5 | 5 | 3 | | 19 | 13 | |
| SS | 7 | 3 | 3 | 3 | | 7 | 9 | |
| ***DRD4*** | | | | | Ns | | | Ns |
| Absence of 7 – allele | 29 | 10 | 5 | 11 | | 29 | 26 | |
| Presence of 7 allele | 11 | 4 | 4 | 3 | | 11 | 11 | |
| ***DRD4/521*** | | | | | 0.05 | | | Ns |
| CC | 14 | 7 | 1 | 2 | | 14 | 10 | |
| TT | 18 | 3 | 4 | 3 | | 18 | 10 | |
| TC | 10 | 4 | 5 | 9 | | 10 | 18 | |
| ***COMT*** | | | | | Ns | | | Ns |
| GG | 12 | 2 | 2 | 8 | | 12 | 12 | |
| AA | 7 | 4 | 1 | 4 | | 7 | 9 | |
| GA | 23 | 8 | 7 | 3 | | 23 | 18 | |

To test if DRD4, DRD4/521, 5-HTT and COMT, attachment security, and the interaction between each polymorphism and attachment predicted physiological response to stress, separate HMLM analyses were performed taking also into account those child, demographic and state factors which were found to be significantly associated with cortisol and alpha amylase levels (see results section of chapter 2). Table 5.3 shows the HMLM results related to the candidate gene polymorphisms, attachment security, and their interaction which were found to significantly predict cortisol and alpha amylase concentrations.

Table 5.3

*Cortisol and alpha amylase levels predicted by genes, attachment classification and interaction between genes and attachment classification. Results of the HMLM analyses*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| | | *Cortisol* | |
| **5-HTT** | 0.34 | **-1.98*** | **1.94*** |
| **Secure Vs Insecure** | 1.56 | -0.91 | 0.50 |
| **5-HTT X Secure Vs Insecure** | 0.06 | 1.07 | -1.05 |
| Birth order | -0.76 | 1.81 | -1.16 |
| Time from awaking to assessment | -2.90** | 3.53*** | -2.95** |
| Duration of trip | 0.73 | 1.47 | -2.35* |
| | | *Alpha Amylase* | |
| **5-HTT** | -1.06 | 1.53 | -1.60 |
| **Secure Vs Insecure** | -1.87 | 1.72 | -1.20 |
| **5-HTT X Secure Vs Insecure** | **2.17*** | -1.03 | 0.73 |
| Time from breakfast to assessment | 0.34 | -2.20* | 1.99* |
| Medication | 0.49 | -2.03* | 1.99* |
| **COMT** | 0.79 | -0.52 | 0.41 |
| **Secure Vs Insecure** | 1.42 | -0.30 | 0.38 |
| **COMT X Secure Vs Insecure** | **-2.38*** | 1.29 | -1.10 |
| Time from breakfast to assessment | 0.82 | -2.61* | 2.39* |
| Medication | 0.97 | -2.09* | 1.94* |
| **DRD4** | 0.70 | **2.00*** | **-2.24*** |
| **Secure Vs Insecure** | -0.54 | **2.21*** | -1.86 |
| **DRD4 X Secure Vs Insecure** | -0.59 | 1.84 | 1.85 |
| Time from breakfast to assessment | 0.06 | -2.21* | 1.83 |
| Medication | 0.67 | -1.71 | 1.51 |
| **DRD4/521** | **-2.03*** | -0.25 | 0.67 |
| **Secure Vs Insecure** | -1.00 | 0.51 | -0.05 |
| **DRD4/521 X Secure Vs Insecure** | 0.53 | 0.44 | -0.66 |
| Time from breakfast to assessment | 0.11 | -2.27* | 2.14* |
| Medication | 0.82 | -1.97* | 1.82 |

The primary variables of interest for this study (genes, attachment and gene-attachment interaction) are typed in bold, as well as their significant effect on physiological measures.

* $p<0.05$; ** $p < 0.01$; *** $p < 0.001$

Considering cortisol first, only the 5-HTT genotype was found to predict both linear and quadratic change in cortisol concentration. Specifically, infants homozygous for the short allele showed a significant cortisol decrease from pre to 20 minutes post the SS, followed by a slight increase at 40 minutes post the end of the stressor compared with infants carrying SL and LL alleles who showed stable cortisol levels across all the three time points (figure 5.1). A trend for 5-HTT to independently predict cortisol levels had already been found for the linear slope in the same direction (see results section of chapter 4).

Interestingly, alpha amylase activity was better predicted by genetic polymorphisms, attachment security, and genetic polymorphism-attachment interactions than cortisol activity was. In particular, each polymorphism was found to be associated with alpha amylase levels, alone or in combination with attachment security. In relation to the gene-attachment interaction, the 5-HTT genotype and the COMT genotype were found to predict baseline alpha amylase levels, but only in interaction with attachment security. Figure 5.2 shows alpha amylase levels across the three time points in infants at no risk (encompassing secure infants with genotype not at risk, secure infants with genotype at risk, and insecure infants with genotype not at risk) and infants with both risks (insecure infants with genotype at risk). As shown in panel A of figure 5.2, infants homozygous for the short allele of the 5-HTT gene who were also insecurely attached to their mothers (both risks) showed higher alpha amylase levels at the baseline than infants who were not at risk for 5-HTT genotype and/or attachment status (no risk). With regard to the COMT-attachment interaction, infants with the met allele of COMT who were also insecurely attached (both risks) showed lower baseline alpha amylase levels than infants who were not at risk for both factors (no risk) (Figure 5.2, panel B).

Further, DRD4 and attachment security were found to independently predict linear and quadratic change in alpha amylase activity (though the interaction was not significant). As shown in figure 5.3 (panel A), infants with the 7 repeat allele had a higher A-A increase from pre to 20 min post the SS followed by a slight decrease at 40 minutes after the end of the stressor than infants without the 7 repeat allele who conversely had a slight increase from pre to 20 minutes post the SS followed by a marked increase at 40 minutes; similarly, infants who were insecurely attached had a higher increase of A-A levels from pre to 20 post the SS than infants who were securely attached (Figure 5.3, panel B). Although DRD4 and attachment security did not significantly predict alpha amylase data when they were considered separately (see results section of chapter 4 and 3, respectively), the same direction of effects were found. In order to verify if attachment security in interaction with the polymorphism contributed to make both effects visible, the HMLM analysis was run again with the same variables but without entering the DRD4- attachment security interaction into the model; coherently, DRD4 and attachment security no longer predicted alpha amylase response to stress.

Last, infants with DRD4/521 CC genotype had higher baseline A-A levels than infants with CT and TT genotype as already emerged in the previous chapter (see results section of chapter 4), whereas attachment security as well as the DRD4/521-attachment interaction did not predict A-A data.

Figure 5.1

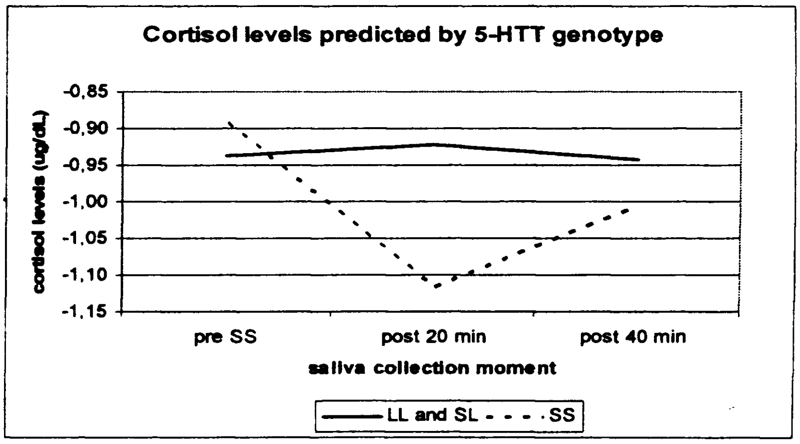
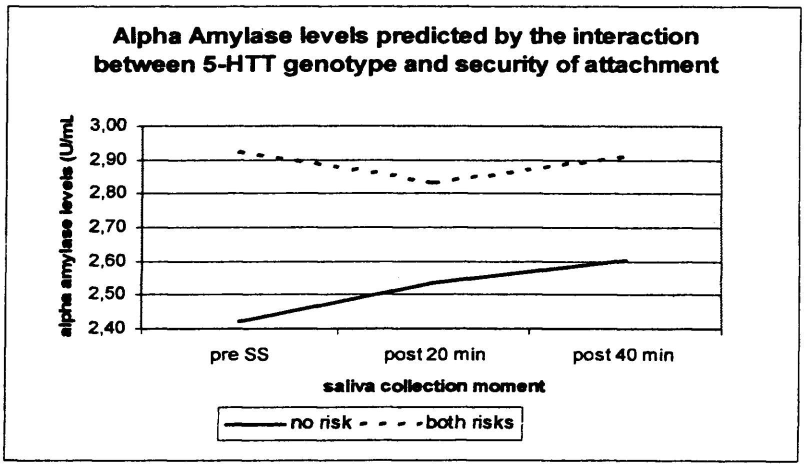



Figure 5.2

Panel A




Panel B

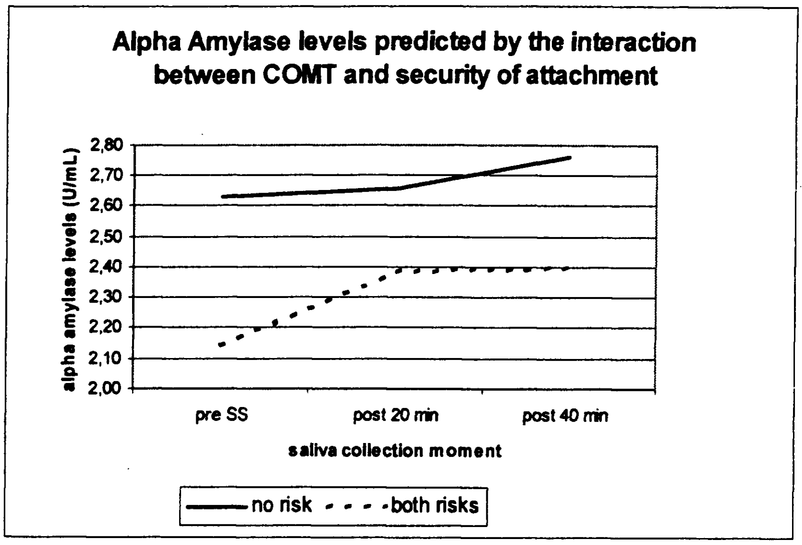
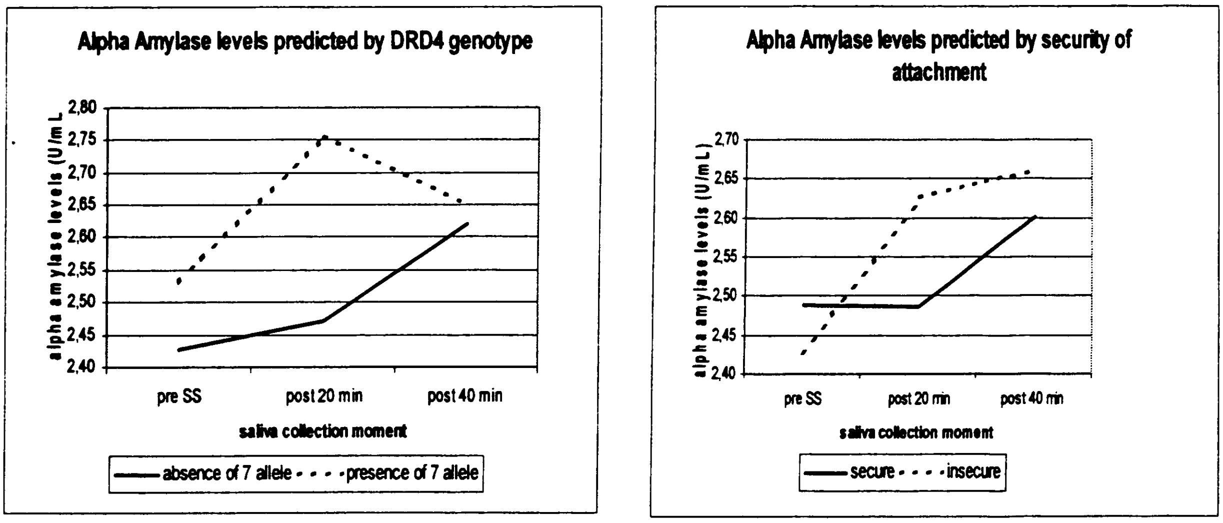


Figure 5.3

Panel A Panel B




*Temperament- attachment interactions on physiological response to stress*

To test for the presence of associations between attachment security and temperament, separate one-way ANOVA analyses with security of attachment (secure vs insecure) as the independent variable and temperament scale scores as the dependent variables were performed. No significant effect of attachment security on temperament traits emerged with the exception of Rhythmicity; specifically, infants with insecure attachment had higher scores on this scale than secure infants both for participants with cortisol ($F$ = 8.24; $p$ = 0.01) and AA data ($F$ = 8.85; $p$ = 0.004). Similarly, infants classified according to the four (B, A, C, D) attachment groups did not differ in temperamental traits with the exception of Rhythmicity scale: specifically, infants with disorganized attachment had higher scores on this scale than infants with secure attachment (cortisol data: $F$ = 3.59; $p$ = 0.02; alpha amylase data: $F$ = 3.43; $p$ =0.02).

To test if temperament traits, attachment security, and the interaction between each temperament trait and attachment predicted physiological response to stress, separate HMLM analyses were performed taking also into account a) those child's demographic and state factors which were found to be significantly associated with cortisol and alpha amylase levels (see chapter 2), and b) those demographic factors (gender, age and SES) which were found to be related to some temperament scales scores (rhythmicity, approach, threshold) (see chapter 4).

Table 5.4 shows the results of the HMLM for cortisol. Only those analyses in which attachment, temperament or their interaction were significant are presented for the sake of space.

Table 5.4

*Salivary cortisol levels predicted by attachment classification, temperament and interaction between temperament and attachment classification. Results of the HMLM analyses*

| | Intercept T | Linear Slope T | Quadratic slope T |
|---|---|---|---|
| **Activity** | -0.72 | -1.01 | 1.32 |
| **Secure Vs Insecure** | -0.69 | -1.61 | **2.01*** |
| **Activity X Secure Vs Insecure** | 1.03 | 1.48 | **-1.97*** |
| Birth order | -0.45 | 2.34* | -1.78 |
| Time from awaking to assessment | -2.82** | 3.77*** | -3.19** |
| Duration of trip | 0.29 | 0.80 | -1.50 |
| | | | |
| **Approach** | 0.55 | 1.72 | **-1.96*** |
| **Secure Vs Insecure** | 0.44 | 0.44 | -0.60 |
| **Approach X Secure Vs Insecure** | 0.01 | -0.72 | 0.78 |
| Birth order | -0.66 | 2.01* | -1.35 |
| Time from awaking to assessment | -3.08** | 3.74*** | -3.05** |
| Duration of trip | 0.66 | 1.62 | -2.53** |
| | | | |
| **Intensity** | **-2.32**** | 1.16 | -0.59 |
| **Secure Vs Insecure** | **-2.45**** | 0.81 | -0.19 |
| **Intensity X Secure Vs Insecure** | **2.89**** | -0.98 | 0.27 |
| Birth order | -0.31 | 2.08* | -1.49 |
| Time from awaking to assessment | -3.11** | 3.52*** | -2.86** |
| Duration of trip | 0.72 | 1.14 | -2.00* |
| | | | |
| **Persistence** | 0.20 | -1.82t | 1.65 |
| **Secure Vs Insecure** | 0.82 | **-2.11*** | 1.71 |
| **Persistence X Secure Vs Insecure** | -0.56 | **2.01*** | -1.66 |
| Birth order | -0.52 | 2.19* | -1.52 |
| Time from awaking to assessment | -3.02** | 4.01*** | -3.30*** |
| Duration of trip | 0.48 | 0.90 | -1.72 |

The primary variables of interest for this study (temperament, attachment and temperament-attachment interaction) are typed in bold, as well as their significant effect on cortisol data.
* p<0.05; ** p < 0.01; *** p < 0.001

The temperamental characteristics of Activity, Intensity, and Persistence were found to predict cortisol levels in interaction with attachment security, as shown by the significant T values for the quadratic slope (Activity x Attachment), the intercept (Intensity x Attachment), and the linear slope (Persistence x Attachment).

Figure 5.4 illustrates the interaction between Attachment and Activity in relation to quadratic change in cortisol. Among infants who were insecurely attached, those with low scores on the Activity scale had a marked decrease of cortisol levels from pre-to 20 min post the SS followed by a slight increase at 40 minutes in comparison with infants who scored high on Activity who showed a slight decrease of cortisol levels from pre to 20 and 40 min post the stressor.

Figure 5.5 shows the effect of the interaction between Intensity and attachment on cortisol baseline levels. Specifically, infants who were insecurely attached and had high scores on the Intensity scale had higher cortisol baseline levels than secure infants with high scores on this scale. However, although insecure and more temperamentally intense infants had the highest cortisol baseline concentrations, secure and less intense infants had higher cortisol baseline levels than secure and more temperamentally intense infants.

As shown in figure 5.6, insecure infants with low scores on the Persistence scale had a deep cortisol decrease from pre to 20 and 40 min post the SS in comparison with infants from the other three groups who showed a less marked decrease of cortisol concentrations.

In order to test which interactions, among those found to be associated with cortisol data, had significant independent effects on stress response, the three temperament scales (Activity, Intensity, and Persistence), attachment security and the three temperament-attachment interactions were entered together into the HMLM model jointly with the covariates. All the previous effects were either significant (attachment x intensity), or near significant (activity and persistence interactions both $p = .06$).

Figure 5.4

*Cortisol levels before and after the SS predicted by the interaction between Activity and attachment security*

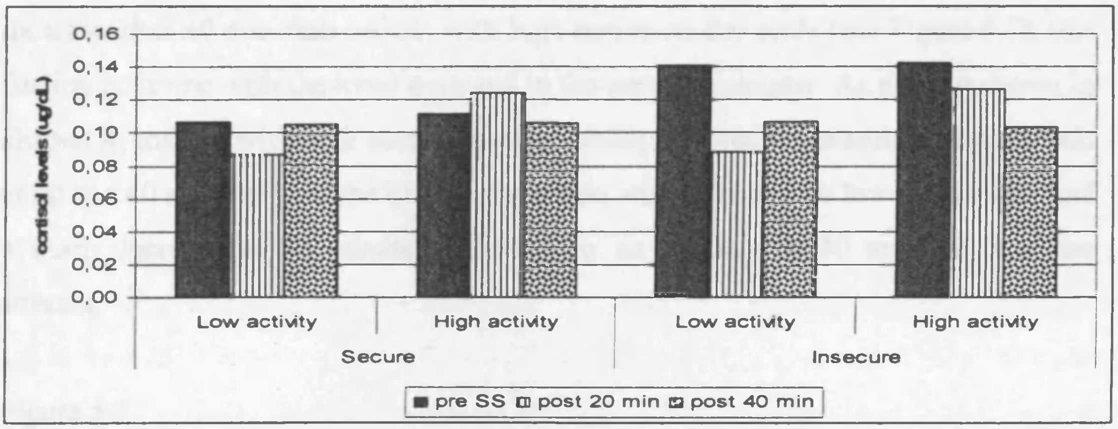



Figure 5.5

*Cortisol baseline levels predicted by the interaction between Intensity and attachment security*

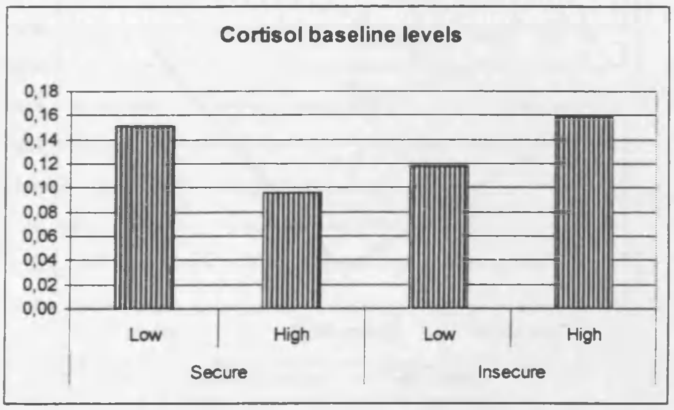
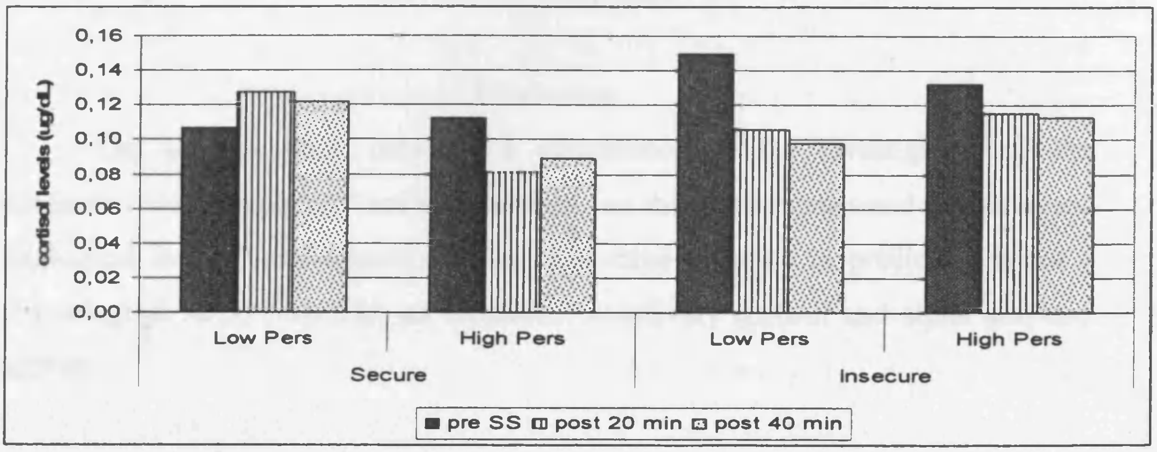



Figure 5.6

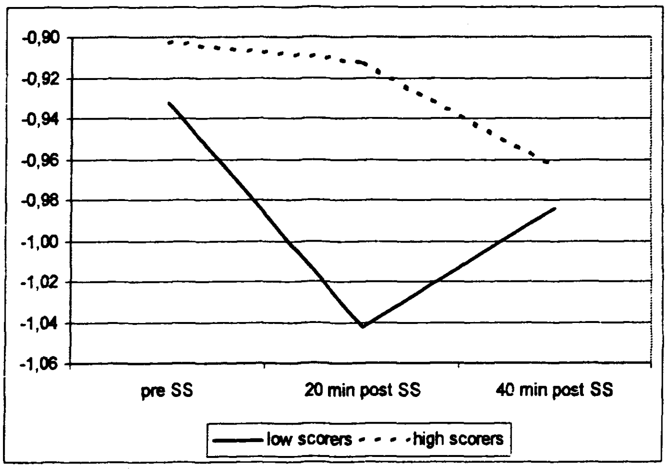
*Cortisol levels before and after the SS predicted by the interaction between Persistence and attachment security*



Last, Approach scale scores and Adaptability scale scores significantly predicted HPA response. Specifically, infants with low scores on the Approach scale had a significant decrease of cortisol levels 20 min after the SS followed by a slight increase after 40 min than infants with high scores on this scale (see Figure 5.7); this finding is in line with the trend emerged in the previous chapter. As already shown in chapter 4, infants with high scores on Adaptability showed decreasing cortisol levels at 20 and 40 minutes post the SS in comparison with infants with low scores who had a sharp decrease at 20 minutes followed by an increase at 40 minutes after the stressor.

Figure 5.7

*Cortisol levels (in μg/dl) predicted by Approach scale scores.*




Finally, no significant temperament-attachment interaction predicted alpha amylase response to the SS. The only significant predictors of alpha amylase data, namely Approach and Inattention, have already been reported in chapter 4.

## Discussion

The present study provided a contribution to the investigation of the interaction between biology and environment - as indexed by genes and temperament (biological factors) and attachment (environmental factor) - in predicting infant's physiological stress responses as measured by salivary cortisol and alpha amylase activity.

*Gene- attachment interactions on physiological response to stress*

This study presents initial evidence for a gene-environment interaction in predicting SAM activity in infancy, as indexed by salivary alpha amylase. However, before discussing the main findings, a brief comment on the preliminary analyses aimed at investigating possible associations between genes and attachment is needed in the light of the current debate about the antecedents of attachment disorganization (i.e. Green and Goldwin, 2002; Madigan et al., 2006).

Each investigated genetic polymorphism (DRD4, DRD4/521, COMT and 5-HTT) was not associated with attachment status when attachment was grouped into secure versus insecure. However, a significant association between DRD4/521 and attachment disorganization was found when all the four attachment classifications were taken into account: specifically, infants with the CT variant were more likely to display disorganization of attachment. Although Lakatos et al. (2002) found a significant effect of the presence of the - 521 T allele on infant's attachment disorganization only in interaction with the DRD4 7 - repeat allele (which were not investigated together here because it was beyond the aims of this study), our finding is likely to support the involvement of DRD4/521 T variant in disorganized attachment and, more generally, the role of altered functioning of dopaminergic neurotransmission in infants with this attachment pattern. Similarly to previous replication attempts (Bakermans-Kranenburg and Van IJzendoorn,2004; Gervai et al., In Press ; Van IJzendoorn and Bakermans-Kranenburg, 2006), the present study failed to confirm a direct link between DRD4 and infant attachment disorganization found in the pioneering studies of the Hungarian group (Gervai et al., 2005; Lakatos 2000; 2002). Not surprisingly, 5-HTT was not found to be associated with attachment status in line with Lakatos and colleagues' study (2003). The COMT polymorphism was also not associated with attachment.

The main finding of the study related to the effects of the interaction between genetic polymorphisms and attachment status on alpha amylase baseline levels. In particular, the current investigation supports the involvement of the 5-HTT and COMT genes in gene-environment interactions, in line with other studies (Caspi et al., 2003; 2005; Fox et al., 2005; Kaufman et al., 2004; Jabbi et al., 2007; Thapar et al., 2005), and provides a first contribution to the understanding of the multifaceted determinants of alpha amylase functioning, as a marker of SAM activity. In fact, despite 5-HTT, COMT and attachment insecurity have not been found to be independently associated with SAM activity (see results section of chapter 3 and 4), when the presence of the two risk genotypes (SS for 5-HTT and AA for COMT) was combined with attachment insecurity two different patterns of outcomes emerged. Specifically, the presence of the 5-HTT short-short alleles in interaction with attachment insecurity was associated with higher alpha amylase levels at baseline. In other words, infants with the short-short variant of the 5-HTT had a greater activation of the SAM system upon arrival to the laboratory, than infants with the other variants (SL and LL) of this polymorphism, but only when they also had an insecure attachment relationship. Consequently, for the first time, the role played by the short form of the 5-HTT gene polymorphism in interaction with an insecure attachment relationship has found to be associated with an hyper-activation of the SAM system in human infants. In fact, although other studies have found that an interaction between this polymorphism and different environmentally stressful conditions confer risk for emotional distress and symptoms in humans (Caspi et al., 2003; Fox et al., 2005; Kaufman et al., 2004), just one study has specifically investigated the interaction between the serotonin transporter gene promoter and attachment relationships in predicting physiological response to stress in animals (Barr et al., 2004). In this study, Barr and colleagues (2004) found that among rhesus monkeys with the short variant of the 5-HTT those exposed to adverse experience early in life demonstrated a marked activation of the neuroendocrine stress axis in response to the stress of separation. Therefore, although in the present study the 5-HTT – attachment interaction predicted SAM activity instead of the HPA response to the stressor of separation, its involvement in explaining a hyper-activation of stress-related systems may also be important in humans.

In contrast, infants with the AA variant (met-met) of the COMT gene polymorphism who were also insecurely attached displayed lower alpha amylase baseline levels than infants with no or one of the two (genetic or environmental) risks. A low activity of both SAM and HPA systems, as indexed by salivary alpha amylase and cortisol, is well documented in children and adolescents with externalizing disorders (Gordis et al., 2006; King, 1998; McBurnett et al., 2000; Moss et al., 1995; Oosterlaan et al., 2005; Raine, 2005). Furthermore, a low enzymatic activity of COMT associated with the Met allele was also found to be related to externalizing behaviours (i.e. Volavka et al., 2004). Furthermore, insecure attachment relationships have often been associated with later externalizing problems (i.e. Sroufe et al., 2005).

Therefore, it can be speculated that insecurely attached infants with the met variant of the COMT gene may be at higher risk of a hypo-activation of SAM system, which might in turn be predictive of later externalizing disorders. Put the other way around, attachment security might be a relevant protective factor for buffering the effects of SAM hypo-activation in infants with an unfavourable genetic predisposition. However, longitudinal studies with larger samples are needed in order to provide further empirical support to this hypothesis.

Other 'minor' findings of the present study are related to the independent effects of the DRD4 genetic polymorphism and attachment status on alpha amylase reactivity to the stress of separation, whereas the effects of the 5-HTT and DRD4/521, on cortisol and alpha amylase data respectively, have already been discussed in the previous chapter. Infants with the 7 repeat allele showed alpha amylase reactivity from pre to 20 minutes post the strange situation followed by a decrease at 40 minutes compared to infants without the risk genotype. Similarly, infants who had an insecure attachment relationship had a significant alpha amylase increase at 20 minutes post the stressor in comparison with securely attached infants. In both cases, the direction of the effect is in line with the theoretical expectations which suppose genetic predisposition (i.e. Linkowski et al., 1993; Oswald et al., 2004) and attachment insecurity (i.e. Nachmias et al., 1996; Spangler and Schieche 1998; Spangler and Grossman, 1993) to play a significant role in infants' inability to cope with stressful circumstances.

To sum up, the main findings of the present study support the idea of genetic vulnerability which, in combination with an insecure attachment relationship, leads to individual differences in physiological stress-related systems. Alternatively, the data may be interpreted as suggesting that secure attachment relationships somehow confer resiliency to individuals who carry alleles that may otherwise increase their risk for adverse physiological outcomes (Suomi, 2006).

*Temperament - attachment interactions on physiological response to stress*

In the current study, interactions between temperamental traits and attachment status have been were investigated in relation to HPA and SAM activity in infancy. However, before discussing the main findings, the preliminary analyses aimed at investigating possible associations between temperament and attachment will be briefly reviewed.

Although the relations between temperament and attachment security have been debated for many years (e.g. Calkins and Fox 1992; Kagan, 1984; Mangelsdorf et al., 1990; Mangelsdorf and Frosch, 1999; Sroufe, 1985), the two constructs are considered to be independent and largely determined, respectively, by genes and environment. In the present investigation temperamental variations were not found to be significantly associated with attachment status in line with attachment research (Nachmias et al., 1996; Sroufe, 1985), with the exception of the dimension of Rhythmicity, where insecure infants showed more problems in the regulation of their biological rhythms than secure infants. Specifically, infants with a disorganized attachment relationship were described by their mothers as more irregular in their sleeping, feeding and evacuation habits than secure infants. Therefore, although the direction of the association between rhythmicity and attachment could not be established because of the cross-sectional nature of this study design, it is likely that some temperamental traits (such as rhythmicity) and attachment security are interrelated though perhaps not interchangeable constructs.

The main findings of the present study are related to the effect of some interactions between temperamental traits and attachment on HPA axis activity, whereas no temperament traits interacted with attachment status in relation to SAM activity. Specifically, three temperament traits (namely Activity, Intensity and Persistence) interacted with attachment status to significantly predict cortisol data. Among these three interactions, the interaction between Intensity and attachment had the strongest effect on cortisol data and was the most robust independent predictor of HPA activity when they were all entered into the HMLM model analyses. It was found that more intense infants, characterized by the display of intense responses (i.e. "he/she shows strong reactions (cries, screams) to failures"), had higher cortisol baseline levels but only when they were also insecurely attached. In other words, as expected from theoretical arguments, attachment security appeared to work as a social buffer against the less adaptive temperamental predisposition of Intensity in regulating HPA functioning. This finding is in line with data from other studies which have investigated the interaction between temperament and attachment in predicting HPA activity (i.e. Nachmias et al., 1996; Spangler and Schieche, 1998; Schieche and Spangler, 2005), although: 1) they focused on another maladaptive temperament trait, namely behavioural inhibition, and 2) they found it to determine cortisol *reactivity* (instead of cortisol baseline levels). However, despite the fact that an interaction between behavioural inhibition and attachment in HPA stress response was not found in the present study, a significant effect of behavioural inhibition (characterized by high scores on the Approach scale) was found, such that behaviourally inhibited infants showed a less marked cortisol decreasing in response to the stress of separation in comparison with uninhibited infants emerged. As this effect becomes evident only when the approach-attachment interaction was entered into the HMLM model analyses, it is possible that this effect is a statistical artefact. Replication with a larger sample would be valuable in order to establish the robustness of this effect.

With regard to Activity-Attachment and Persistence-Attachment interactions which were found to predict cortisol reactivity in response to stress, the data were not so easily interpretable. Considering Activity-Attachment interaction first, it was found that among insecure infants, those characterized by high levels of motor activity had a less marked cortisol decrease from pre to post the SS in comparison with those who displayed low levels of activity. Similarly, insecure infants characterized by good persistency, in terms of amount of time spent on the same activity (e.g. "When he/she learns a new ability (throwing, drawing), he/she practices it for ten minutes or more") had the most marked decrease of cortisol levels from pre to post the SS when compared with the other groups.

No temperament-attachment interactions were associated with alpha amylase activity in response to the stress of separation, thus confirming the apparent independence of the two salivary physiological markers of HPA and SAM functioning already emerged (see results sections of chapters 2, 3, and 4).

To sum up, for the first time in human infants, this study has shown how some gene and temperament characteristics may interact with attachment to contribute to individual differences in HPA and SAM activity. Interestingly, the two kind of interactions investigated, gene x attachment and temperament x attachment, were found to impact on salivary alpha amylase and cortisol levels respectively. Specifically, attachment status in interaction with some genes tended to be associated with SAM activity functioning whereas HPA activity was associated with interactions between attachment and some temperamental traits. However, the present findings represent just a first step in understanding the role played by biology-environment interplay in explaining infant's physiological stress response.

Future studies based on larger samples are needed in order to replicate our data, as well as to better explore the contribution of the interaction between the 4 attachment patterns and the all genotyping of the investigated genes in determining how infants physiologically react to challenging circumstances. Moreover, a better measure of temperament, based on direct observation of infant behaviour rather than on parent report, might probably contribute to more rigorously assess data on temperament-attachment interactions.

# CHAPTER 6

# FINAL DISCUSSION

The main aim of the research presented in this thesis was to investigate if and how biological (genes and temperament) factors and social experiences (child-mother attachment relationship) are associated, separately and combined, with the stress response of separation in infants. In this last chapter, after a general reiteration of the rationale of the study and a brief overview of the hypotheses, the main findings will be summarized. Particular emphasis will be given to those theoretical and methodological explanations which can help to better understand both negative and positive findings. Last, a concise section about the future directions in this field will conclude the present dissertation.

*The rationale of the study*

The importance of understanding which environmental and biological factors are involved in determining individual differences in physiological responses to stress is widely recognized, as the impact of stress on physical and mental health cannot be ignored (e.g. Bremner, 2003; Bremner and Vermetten, 2001).

From a biological perspective, stress responses are composed of the activation of neurobiological systems that help to preserve viability through change or allostasis (McEwen and Seeman, 1999). Stress responses are affected by two distinct but interrelated systems: the hypothalamic-pituitary-adrenocortical (HPA; Stratakis and Chrousos 1995) system and the sympathetic-adrenomedullary (SAM; Frankenhaeuser 1986) system. The first involves activation of the hypothalamic–pituitary–adrenal (HPA) axis and the secretion of cortisol into circulation. The second, and faster acting component, involves activation of the sympathetic branch of the locus ceruleus/autonomic nervous system (SNS) and the release of catecholamines (Chrousos and Gold, 1992). The majority of the available empirical evidence relevant to testing stress-related responses in child development has focused on the activity of the HPA axis as indexed by individual differences in levels and change in cortisol (for a review see: Gunnar and Donzella, 2002; Gunnar et al, 2007). However, to advance our understanding of the interaction of biological, social, and behavioural processes in childhood, multiple measurements of stress-related biological processes need to be investigated (e.g. Bauer et al., 2002; Donzella et al., 2000; Granger and Kivlighan, 2003). Therefore, researchers have focused their attention on identifying a biomarker of SAM activity which could be easily and non-invasively measured in saliva. A small literature, largely conducted with adult participants, revealed salivary alpha amylase as a viable candidate (Granger et al., 2006). In fact, alpha amylase, an enzyme produced locally in the oral mucosa, has been found to be responsive to stress and its validity as a surrogate marker of sympathetic activity has been supported by several investigations (Chatterton et al., 1996;Granger et al., 2006 in press; Nater et al., 2005, 2006).

Although the stress response can be said to serve a protective function (McEwen, 1998), the chronic mobilization of the stress response also can exert damaging, even pathogenic effects (Bremner, 1999; McEwen & Sapolsky, 1995; Sapolsky, 2000a, 2000b). Early stress has long term effects on brain structures and systems that play an important role in the stress response, including increased activation of corticotrophin releasing factor and the HPA axis and noradrenergic systems (e.g. Bremner and Vermetten, 2001). Early stressors have lasting effects on the hypothalamic-pituitary-adrenocortical axis and norepinephrine systems; other brain systems that are involved include benzodiazepine, opiate, dopaminergic and various neuropeptide systems. These neurochemical systems modulate function in several brain regions, including hippocampus, amygdala and prefrontal cortex. Long-term alterations in these brain regions are hypothesized to play a role in the maintainance of several mental disorders (such as post traumatic stress disorder and depression). In fact, studies in patients with a history of childhood abuse and with depression and PTSD are consistent with long-term dysregulation of the HPA axis and noradrenergic systems.

While it is important to investigate the biological consequences of acute and chronic stress it is also critical to understand which factors contribute to individual differences in HPA and SAM response to mild stress in low risk samples of children. Indeed, there seems to be much greater variability between organisms in the magnitude and quality of response emitted to stressors which are more psychological in nature (Sapolsky, 1994). Without a doubt, the confluence of a number of factors, including physical status, genetic makeup, prior experience, and developmental history, determine the differential ways in which organisms may react to a stressful event (McEwen, 1994c; Sapolsky, 1994). In particular, the combination of previous experience and developmental history may either sensitize or protect the organism from subsequent stressful challenges. Additionally, more long-term stress responsiveness is characterized by interindividual variability and is related, in part, to experiential influences on gene expression (Meaney, Diorio, Francis, Widdowson, LaPlante, Caldji, Sharma, Seckl, & Plotsky, 1996). Reasonably, many researchers believe that our ability to cope with stress originates in infancy through the interaction between our experiences and genes. In attempting to understand how social experiences impact both typical and atypical aspects of emotional development, several studies have focused on the investigation of attachment relationship in infants, taking also into account the effects of some child's internal characteristics, such as maladaptive temperamental traits, in determining physiological reaction to stress. While both the attachment relationship and temperamental traits play a significant role in modulating HPA axis reactivity to the stress of separation from the mother, as indexed by salivary cortisol (e.g. Nachmias et al., 1996; Spangler and Grossman, 1993; Spangler and Schieche, 1998), no published studies had investigated attachment and temperament effects on SAM system reactivity, as indexed by salivary alpha amylase, yet. Moreover, the contribution of genetics in predicting salivary cortisol and alpha amylase response to stress in infants has also not been investigated previously.

In the present study, the child-mother attachment relationship, some genetic polymorphisms (DRD4, DRD4/521, COMT and 5-HTT), and temperamental traits were tested as predictors of both (cortisol and alpha amylase) physiological markers during the Strange Situation (SS) procedure in an Italian sample of around 70 healthy infants aged 12 to 18 months. While HPA and SAM reactivity to the stressor of the Strange Situation were not expected in our low-risk sample on the basis of previous studies an activation of both systems was hypothesized in specific subgroups of infants characterized by restricted ability to cope with stress. Specifically, individual differences in attachment patterns were expected to be associated with the stress response; and security of attachment was hypothesized to act as a moderator of less adaptive temperamental traits and genetic predisposition in HPA and SAM activation.

In the following paragraphs, positive and negative findings of the current study will be discussed with a particular attention to methodological issues.

*HPA and SAM activation in response to stress in human infants*

In the current study no overall salivary cortisol reaction in response to the stress of separation elicited by the Strange Situation procedure was observed, whereas an increase of salivary alpha amylase concentrations from pre to post the Strange Situation was found.

The lack of glucorticoid response to the Strange Situation procedure supports evidence that in humans the HPA system might be comparatively less responsive to stressors over the first year of life (Gunnar et al., 1996; Gunnar and Donzella, 2002; Ramsay and Lewis, 1994). In fact, several researches have reported no significant elevations of cortisol in response to various mildly threatening events that elicit distress, wariness, and inhibition of approach (i.e. inoculations, approach by a stranger, and encounters with strange, novel events) in infants between 6 and 18 months of age (Gunnar et al., 1996; Nachmias et al., 1996; Jacobson et al., 1994; Ramsay and Lewis, 1994; Spangler and Schieche, 1998). These data are in line with studies conducted with rats which have described a "stress hypo-responsive period" shortly after birth during which it is difficult to elevate cortisol to many stressors. It has been suggested that this period serves to protect the developing brain from adverse impacts of chronic cortisol elevations (Gunnar and Donzella, 2002). Although the functional effect seems similar in humans and rodents, because the mechanisms are not understood this should be considered an analogous, not homologous phenomena (Gunnar and Donzella, 2002). However, another possible explanation concerning the absence of HPA reactivity in response to the Strange Situation might be related to the characteristics of this procedure. In other words, it is possible that the Strange Situation does not represent a sufficient stressor to trigger adrenocortical reactivity in infancy. Indeed, although the strange situation was devised to produce a moderate stress in infants, its main aim is that of identifying individual differences in attachment behaviours rather than eliciting stress responses. Moreover, this standardized procedure recommends a) to comfort the child if he/she is distressed by a strange adult female, and b) to curtail the separations episodes if the child gets too distressed (Ainsworth et al., 1978), thus preventing the observation of more intense stress-related reactions. Furthermore, the strange situation deliberately introduces two relatively brief stressors that may not be prolonged enough to yield a reliable stress response. Last but not least, in consideration of the fact that a decline of adrenocortical values during the morning time is expected due to the cortisol circadian rhythm (e.g., Larson et al., 1998; Spangler, 1991), it is possible that such a decline may have masked minor cortisol increases actually triggered by this mildly stressful situation.

In contrast, salivary alpha amylase reactivity in response to a mild stressor in infancy, as the Strange Situation is, was found for the first time in the present study. In fact, while previous studies (e.g. Spangler and Grossman, 1993) have reported an activation of other SAM markers, such as heart rate, in response to the Ainsworth's procedure (Ainsworth et al., 1978), an increase of alpha amylase levels after challenging circumstances has been found in adults (Nater et al., 2005; 2006; Takai et al., 2004; Van Stegeren et al., 2006) and adolescents (Gordis et al., 2006) but has never been reported in infancy (Granger et al., 2007). Consequently, it can be supposed that salivary alpha amylase, compared to salivary cortisol, is a more sensitive physiological measure of the stress response. This result could be due to the more sensitive threshold of reactivity in the SNS (i.e. salivary alpha amylase) than in the HPA axis; theoretically, SNS reactivity would occur to challenges that are more mild and transient than those required to activate the HPA axis (Lovallo and Thomas, 2000). However, another plausible explanation about the heightened SAM activity after the stressor should be taken into account. A recent study carried out by Nater and colleagues (2007) has found that salivary alpha amylase has a distinct diurnal profile pattern, with a pronounced decrease within 60 minutes after awakening and a steady increase of activity during the course of the day. This finding replicates previous data obtained by Rohleder et al. (2004) who had already reported a continuous increase of salivary alpha amylase levels over the course of the day but in a smaller sample. Although the sample of these studies was composed by adult participants and no data are available in relation to the presence of a similar diurnal alpha amylase pattern in infancy, it is not possible to exclude that the activation of SAM system after the Strange Situation is, instead, a sign of the circadian oscillations of salivary alpha amylase activity.

To sum up, the most significant remark concerning salivary cortisol and alpha amylase response to the strange situation is related to the potential interfering effect of circadian rhythm of both stress measures on data. Indeed, it is striking to observe that no study has employed a control group in order to verify if physiological findings are induced by a stress procedure or are instead affected primarily by the circadian rhythm. Therefore, future bio-behavioural studies should include a control group composed by infants assessed in the same way as infants from the experimental group but who are not separated from their mothers during the strange situation, in order to detect the influence of maternal separation as a real stressor able to trigger physiological responses.

*Is attachment relationship a social buffer against stress?*

The role played by the organization of the attachment relationship in buffering the physiological effects of the stress response in infants was one of the main issues investigated in the present study. Attachment research has often emphasized the influence of individual differences in attachment in shaping physiological response to challenges, postulating a better ability of secure infants to cope with stress compared to insecure infants. To support attachment theses, positive findings from bio-behavioural studies are often cited; in these studies it is stressed how the behavioural strategy of securely attached infants during the Strange Situation seems to be more adaptive, as HPA and SNS activation was observed only in insecurely attached or disorganized infants and not in securely attached ones (e.g. Hertsgaard et al., 1995; Spangler and Grossmann, 1993; Spangler and Schieche, 1998; Sroufe and Waters, 1977). On the other hand, inconsistent results and methodological limitations of these investigations are less highlighted in attachment research. In fact, although all existing studies show no physiological reactivity in response to stress in infants with secure attachment, the available evidence shows a) mixed and not unequivocal data concerning the association between insecure attachment patterns and heightened physiological response to separation, and b) a weak ground of the study design and methodology.

Data from the present study did not support previous findings concerning individual differences in adrenocortical response to stress as a function of attachment status, while partial support for the role played by attachment insecurity in determining an activation of the SAM system response to stress is reported.

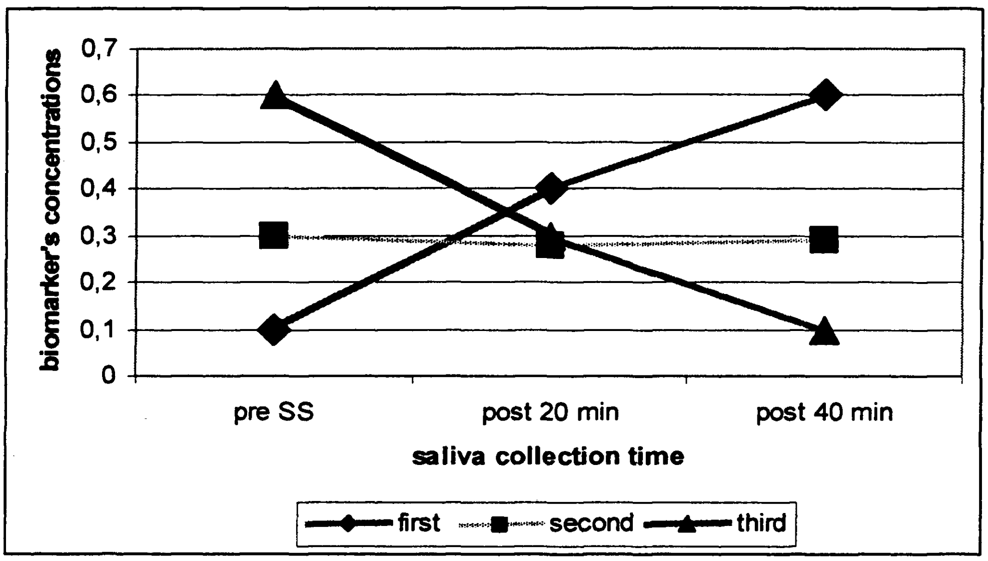
Specifically, in contrast with the model of attachment theory, infants with avoidant attachment showed higher baseline levels and a larger decrease of cortisol levels from pre to post the stressful procedure. In order to better understand this data, two possible explanations should be kept in mind as already described in the third chapter. First, avoidant infants showed high baseline cortisol concentrations which might reveal the presence of adrenocortical activation already at the beginning of the laboratory visit in response to the arrival in the institute or to other factors; the decline, consequently, could represent subsequent recovery during data assessment. Second, the present finding might reveal a potentially dysregulated pattern, termed declivity, reported by recent studies. Specifically, cortisol decreases below not only pretest levels (which might be elevated in anticipation of testing) but also below home basal levels at the same time of day have been found (Shirtcliff et al., 2005; van Goozen et al, 1998). Moreover, a recent study (Blair et al., 2006) has shown higher baseline cortisol and decreasing cortisol levels at 20 and 40 minutes post emotional challenges in infants of mothers exhibiting lower levels of sensitivity in comparison with infants of mothers exhibiting higher levels of sensitivity, who showed the opposite trend. Although the similarity between data from Blair et al. (2006) study and from the present study is notable, there is still too little knowledge about the meaning of declivity to know whether it is associated with disordered functioning (Adam et al., In Press).

If cortisol decreases after the stress of separation in avoidant infants was an unexpected finding, the activation of SAM system in resistant infants in response to the strange situation can be more easily interpreted within the frame of attachment theory. In fact, as expected, infants with resistant attachment showed a stronger alpha amylase increase after the stressor compared to infants with other attachment patterns. Therefore, it is likely that the SAM system can be more easily activated by infants who use a suboptimal strategy to cope with stress, as resistant infants do. The fact that resistant infants did not show a correspondent activation of the HPA axis raises stimulating questions about the characteristics of the relation between this attachment pattern and SAM functioning. Specifically, infants with resistant attachment pattern are characterized by strong distress during separation, ambivalent behaviours towards the parent during reunion, and an inability to re-establish emotional stability for a long period. Such characteristics might be more likely to activate a prompt reaction of the organism from a physiological perspective. In other words, resistant infants might be more sensitive to the activation of the sympathetic nervous system as it fosters fight and flight behaviours (Porges, 1997). However, as resistant infants had lower alpha-amylase baseline concentrations it could also be likely that the activation of the SAM system was more easily triggered. To exclude this possible occurrence, the presence of a control group would have been useful, as already noted in the previous section.

Furthermore, in order to better test our data another study design would have been more useful. Specifically, as in single case study designs the collection of multiple baselines for salivary cortisol and alpha amylase would have helped to better clarify our data. As shown in figure 6.1, a single baseline measure does not allow control for causal inference. Instead, the presence of multiple baseline collections would have helped, for instance, corroborate the presence of cortisol decreases from pre to post stressor in avoidant infants. For example, in order to properly characterize a change in cortisol or alpha amylase, it is highly desirable to demonstrate no directional trend at baseline, or at the very least to well-describe this trend. To do so would require a relatively lengthy baseline period and multiple measurements of the relevant biomarkers. Surprisingly, no bio-behavioural study has employed a similar design to account for individual differences in HPA and SAM reactivity as a function of attachment patterns.

Figure 6.1

*An exemplification of possible outcomes with a single baseline measurement*



Additionally, some unexpected results related to the lack of association between insecure attachment and physiological response raise questions beyond the methodological limitations of attachment studies up to now reported. In contrast with theoretical expectations, a lack of physiological responses in resistant and avoidant infants (who did not show cortisol and alpha amylase response to the stress of separation respectively), and in disorganized infants (who did not show both cortisol and alpha amylase increasing after the stressor) was found in the present study. Other studies have also reported similar conflicting data (Spangler and Grossman, 1998; 2005). In order to account for these inconsistent results, the involvement of other factors which in interaction with attachment status may help to better predict individual differences in stress response was hypothesized. Among these factors, genetics and temperament were expected to play a significant role.

*What is the contribution of biological factors in stress response?*
*The role of gene x environment and temperament x attachment interactions*

Similarly to studies exclusively focused on the environmental antecedents of individual stress response, there are several psychobiological investigations which have independently examined the contribution of biological factors, such as genetics and temperament, in determining variations in physiological response to stress both in animals and humans. Only a few studies, however, have investigated the extent to which individual constitutional characteristics (genes and temperament) and early experiential factors work interactively in shaping differences in the activation of the HPA and SAM systems (e.g. Barr et al., 2004; Nachmias et al., 1996).

In the present study some genetic polymorphisms and temperamental traits were investigated, alone and in interaction with attachment status, as potential predictors of salivary cortisol and alpha amylase response to the stress of separation. Interestingly, a larger number of significant effects emerged when the interactions between attachment and genetics, as well as between attachment and temperament, were taken into account rather than when biological factors were considered independently from attachment status. In general, attachment status in interaction with some genes predicted SAM activity functioning, and in interaction with some temperamental traits predicted HPA activity functioning.

Among genes, the serotonin transporter (5-HTT) and catechol-Omethyltransferase (COMT) genes were found to be associated with alpha amylase baseline activity but only when they interacted with security of attachment. Specifically, insecure infants with the short allele of the 5-HTT gene had a greater activation of the SAM system at baseline while insecure infants with the met allele of the COMT gene had lower alpha amylase baseline levels. Accordingly, hyper- and hypoactivity of the SAM system may be related to the interaction between early social experience, as indexed by insecure attachment, and specific genetic polymorphisms (5-HTT and COMT, respectively). Although these data need to be replicated in larger samples, this is the first study which provides initial evidence of gene x environment interaction on SAM system functioning in infants from a low risk-sample. In fact, no studies have investigated COMT x attachment interaction in shaping HPA and SAM activity functioning, whereas a recent study reported significant data on the role played by 5-HTT x attachment interaction in determining individual differences in physiological stress response (see Barr et al., 2004) but in rhesus monkeys.

As Suomi reported in a recent paper (2006), although it seems likely that significant G x E interactions involving specific genetic polymorphisms and differential early experience occur and have an impact both on behaviour and physiology in humans and animals, it is also true that their evidence is largely statistically based. Consequently, data like those found in the current study can be interpreted according to two different perspectives. One interpretation is that "good genes" somehow confer resiliency to adverse early attachment relationships on those individuals who carry them; on the other hand, another interpretation of the same data is that secure attachment relationships somehow confer resiliency to individuals who carry "bad genes" that may otherwise increase their risk for adverse developmental outcomes. In other words, "good" gene can offer protection from a "bad" environment but it could also be that a "good" environment can protect individuals with "bad" gene. Although these two interpretations are not mutually exclusive and can be taking place in the same individual, further empirical evidence is needed to identify and understand those processes involved in this kind of G x E interactions. However, taking into account the recent studies of Meaney's group which provide the best examples of how environments can have effects on gene expression in rats (Cameron et al., 2005; Champagne et al., 2004; Meaney and Szyf, 2005; Weaver et al., 2004), it can be reasonably speculated that specific early social experiences might modify gene expression also in primates.

Among temperamental traits, the most robust independent predictor of HPA activity was represented by Intensity in interaction with attachment security: infants characterized by the display of intense responses had higher cortisol baseline levels but only when they were insecurely attached; conversely, highly intense infants who were securely attached showed lower cortisol levels at the baseline. Therefore, as expected, this finding suggests that secure attachment pattern is likely to function as a buffer against HPA hyperactivity in infants with highly intense temperamental dispositions. Also, it can be interpreted in the light of what said before that "good" environment can protect individuals with "bad", largely genetically determined, temperamental traits, at least for this specific temperamental characteristic. In fact, other data from the present study related to attachment x temperament interactions do not entirely support this hypothesis. Specifically, attachment security did not seem to work as a protective factor against stress, as indexed by cortisol reactivity, in infants with other maladaptive temperamental traits (high levels of motor activity and low ability to persist on activities). As a result, the role of attachment security as a significant strategy a child has to reduce the activation of the HPA system in response to stressful circumstances, even in the presence of less adaptive temperamental characteristics which might bias him/her, is not always supported in this study.

To sum up, in the present study HPA and SAM activity was predicted by a larger number of gene x environment interactions in comparison with the number of separate biological (genes and temperament) and environmental (attachment patterns) factors. Therefore, the main result is related to the fact that in many cases the effects of genetics and temperament may be contingent upon the co-action of environmental risks. In fact, this study generally supports the idea of genetic vulnerability (Rutter, 2006) which, in combination with an environmental risk factor (insecurity of attachment), leads to physiological hypo- or hyper-activation of the HPA and SAM systems. In other words, genetic and environmental risk factors may increase the probability of negative developmental outcomes multiplicatively (Seifer et al., 1992). Another possible interpretation of this kind of data is provided by Belsky 's "differential susceptibility model" (1997, 2005). According to Belsky, some individuals have traits and developmental routes that are more fixed by their genetic endowment, whereas others are more plastic and susceptible to rearing influences. For evolutionary reasons, these latter individuals are more malleable and responsive to the features of early childhood environments and adjust their biobehavioural development accordingly. Belsky argues that children with a ''difficult'' temperament seem, intriguingly, most susceptible to the effects of rearing. Specifically, infants with a difficult temperament may be more susceptible to environmental influence that may have both negative and positive outcomes, depending on whether they are raised in less or more supportive environments respectively. Recently, empirical evidence has been accumulating in supporting Belsky 's model of differential susceptibility. In particular, a study by Gilissen and colleagues (2007) is particularly relevant for the present investigation, as it tested Belsky's model assessing children's physiological reactions to stress as the outcome. The authors assessed skin conductance and heart rate variability in response to fear-inducing and neutral film clips in children aged 4- and 7- year-olds. They found that attachment security affected the reactivity to fearful stimuli in temperamentally more fearful children but not in less fearful children. Specifically, more temperamentally fearful children with less secure relationships showed the highest skin conductance reactivity to the fearful film clip, whereas comparable children with more secure relationships showed the lowest skin conductance reactivity. Consequently, as more fearful children were more influenced by the quality of the attachment relationship (positively and negatively), the study lends more evidence for the differential susceptibility hypothesis and less support to the concept of cumulative risk.

The differences between the cumulative model and the susceptibility model are relatively subtle (e.g. the susceptibility model stressing the greater responsiveness of susceptible children to positive rearing experience) and the current study does not provide strong evidence for one model over the other. For example, the statistically significant interaction between the temperamental trait of intensity and attachment in predicting cortisol baseline level (see chapter 5) could be interpreted to support both the cumulative risk and Belsky's model. In fact, we found that infants who were more temperamentally intense had the highest cortisol baseline levels when they also were insecurely attached, which could lend support to the cumulative model. However, when more temperamentally intense infants had a secure attachment relationship with their mothers they showed the lowest cortisol baseline levels, thus supporting Belsky's hypothesis of a greater susceptibility of temperamentally "difficult" children to positive rearing. Arguably, lacks of statistical power to discriminate between the two, as the models make relatively subtle differences in prediction regarding the patterning of the means. What is certainly true, and potentially useful, is that the two models emphasize different kinds of causal mechanisms that could be explored in future research, a point that is considered in greater detail below.

*Future directions*

This study represents a first step in the direction of a better understanding of how biological and environmental factors are involved in the stress response in infancy as well as their complex interplay. From a theoretical perspective, the importance of identifying which factors play a role in determining individual differences in physiological responses to mild stressors in infancy can provide significant clues to distinguishing resilient from vulnerable individuals. In fact, behavioural and physiological resilience might in part develop from infants' and young children's experience coping with the inherent normal stress of daily life and social interaction (Tronick, 2006). On the other hand, investigating the risk factors associated with anomalous HPA and SAM functioning in this developmental period, as well as the consequent impact of physiological stress responses on the developing brain, may be of particular note; indeed, these kind of investigations can help to better understand the mechanisms which heighten the risk of emotional and behavioural problems later in the development (Gunnar, 2000; Heim and Nemeroff, 2001). Moreover, despite the limitations discussed throughout the whole dissertation, the present study has several methodological strengths among which a) the investigation of both the stress-related systems, as indexed by multiple measures of salivary cortisol and alpha amylase levels, and b) the careful control of those socio-demographic and child's state (e.g. time of day, medications, sleeping and feeding) factors which interfered with cortisol and alpha amylase data.

To conclude, several questions clearly require further investigation in future psychobiological studies which should focus on various challenges concerning the antecedents of the stress response, the multifaceted response of the physiological stress-related systems, and methodological issues.

Considering the antecedents of the stress response first, two significant challenges for the next generation of studies are: a) to understand the mechanisms and processes of G x E interactions, and b) to identify better measurement strategies for characterising the environment in order to discover G x E interactions. Specifically, the next generation of biosocial research needs to go beyond simple description of interaction effects and research the fundamental mechanisms and processes underlying them (Raine, 2002). In fact, it seems increasingly likely that individual differences in physiological resilience to environmental adversity represent the product of complex interactions among multiple genes and characteristics of the physical and social environments within which development takes place. Identifying, characterizing, and understanding the basis for such complex interactions certainly represents a considerable challenge for future research. Similarly, further studies are needed to detail the developmental processes through which genes interact with other biological constituents and with the environment in shaping infant's stress response. For example, because statistical interactions between two variables (indices of genes and environments) are essentially symmetric in nature it is not clear in most studies (including the present one) whether genes moderate the effects of environments or viceversa. The issue is not trivial, because different models of moderation lead to different kinds of putative mechanisms and different kinds of avenues of inquiry. For example, if genetic factors moderate parenting-based risks then it might be speculated that certain genes leave individuals less responsive to parental input. Thus, this might lead researchers to focus on understanding which parental behaviours impact on physiology and therefore why certain genes might make those factors less significant. Alternatively, if parenting moderates genetic risk, then this would lead researchers to explore what some of the genetically based characteristics there are that parents are somehow reacting to, and altering. E.g. is it that genetically at-risk babies are prone to strong emotional reactions, and therefore this can be offset by parents who help their babies manage those reactions? This in turn might reduce the impact of the genetic risk on actual physiological responding. By outlining different mediating mechanisms the question of what moderates what (genes moderating environments or environments moderating genes) can become more than just a question of language.

Moreover, several researchers have highlighted the need to focus on better measurement of the environment as it seems to be crucial to the discovery of G x E interplay even in samples of modest size (Rutter, 2003; Wong et al, 2003). There are two kinds of evidence which support this approach in future studies. First, some multifactorial disorders and organism's complex responses (as physiological stress-related responses are) are likely to be the result of variations in a relatively small number of genes whose effects are conditional on exposure to environmental risk (Caspi et al., 2003). Second, the magnitude of the impact of measurement precision on power to detect gene-environment interaction on continuous traits would suggest that smaller studies with better measurement may be preferable to very large studies with less precise measurement (Wong et al., 2003).

As far as the investigation of physiological stress response is concerned, an important area which will require the attention of researchers is that of the understanding of the causes and consequences of the frequent asymmetry between the two stress systems found in several psychobiological studies. In fact, in spite of the fact that the two stress response systems are biologically intertwined, studies which have employed measures of both HPA and SAM system, such as salivary cortisol and alpha amylase, have found opposite patterns of outcome (e.g. Gordis et al., 2006; Granger et al., In Press). More information about what conditions might produce asymmetric effects in the different stress response systems and what the consequences could be of such asymmetry would shed light on how these systems work. Moreover, the non invasive means of assessing both the HPA and SNS via saliva should enable researchers interested in understanding how social and psychological states influence the coordination of these stress systems to do so not just in the laboratory but in the context of everyday life and/or within the same individuals cross-situationally or on repeated occasions.

Another challenge for future human neuro-endocrine stress studies is represented by salivary alpha amylase as a reliable marker of SAM activity. Although it seems to be a good measure of stress response, additional methodological research is needed for a better understanding of the advantages and disadvantages of salivary alpha amylase levels in comparison to already established markers of SAM system activity. Moreover, further investigations will be needed to corroborate our data about the interfering effects of factors such as feeding and medication, on salivary alpha amylase activity. Future studies should also examine whether the response in alpha amylase to the Strange Situation generalizes to other stressors in infancy.

Last but not least, results of this study suggest several avenues for addressing methodological issues in psychobiology research. First, future studies aimed at studying individual differences in physiological reactions to stress should employ control groups in order to check for diurnal variations of most of biological parameters of stress. Second, multiple baselines of physiological measures should be collected with the aim to reliably detect a baseline, and therefore avoid misleading interpretations about the post-challenge reactions. Third, larger and longitudinal samples will be needed to better detect how individual differences in genetic make up and early social experiences may affect short and long-term reactions to stress along development both in low and high risk samples. Related to this, research with physiological measures on dyads with not yet established or extremely insecure or disordered attachment relationships (e.g. institutionalized settings, foster care, adoption) may reveal more information about the psychological meaning of these physiological responses.

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